VIVIANA VILAR DA SILVA

ANTITUMOR ACTIVITY OF XANTHONE DERIVATIVES: EFFECTS ON THE IMMUNE MICROENVIRONMENT

Dissertação de Candidatura ao grau de Mestre em Oncologia (Especialização em Oncologia Molecular) submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador:
Doutor Rui Medeiros
Professor Associado Convidado de Ciências Médicas
Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal.

Co-orientadores:
Doutora Fátima Cerqueira
Professora Associada de Ciências Médicas
Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal.

Doutora Madalena M.M. Pinto
Professora Catedrática em Química Orgânica e Farmacêutica
Faculdade de Farmácia, Universidade do Porto, Porto, Portugal

This work was developed in the Center of Investigation in Biomedicine of Fernando Pessoa – Energy, environment and health research unit (CEBIMED of FP-ENAS), in Laboratório de Química Orgânica e Farmacêutica, Departamento de Ciências Químicas, Faculdade de Farmácia da Universidade do Porto, and in the Centro de Química Medicinal da Universidade do Porto – CEQUIMED-UP and was funded through national funds from Fernando Pessoa Fundation, FCT – Fundação para a Ciência e a Tecnologia under the project CEQUIMED – PEst-OE/SAU/UI4040/2014, FEDER, POCI, POPH/FSE/QREN.

Agradecimentos

Não posso deixar de agradecer a quem direta ou indiretamente colaborou para a realização deste trabalho e que, assim, contribuiu para o meu crescimento tanto profissional como pessoal:

Ao Instituto de Ciências Biomédicas Abel Salazar, na pessoa do Digníssimo Diretor Professor Doutor António Sousa Pereira, e à coordenação do mestrado de Oncologia, na pessoa da Professora Doutora Berta Silva, pela oportunidade de aprofundar os meus conhecimentos ao longo destes dois anos de mestrado.

À Universidade Fernando Pessoa, na pessoa do Digníssimo Reitor Professor Doutor Salvato Trigo, pelas condições de trabalho que me proporcionou.

Ao Professor Doutor Rui Medeiros, agradeço a orientação e a oportunidade que me proporcionou, a simpatia com que sempre me recebeu e todos os conhecimentos que me transmitiu.

À Professora Doutora Fátima Cerqueira, minha coorientadora, o meu sincero agradecimento pelo apoio constante, pela disponibilidade, pela amabilidade e pelos indispensáveis conselhos que forma determinantes na realização deste trabalho.

À Professora Doutora Madalena Pinto, pela coorientação, porque me permitiu integrar este projeto, obrigada por toda a sabedoria que me transmitiu e pela enorme simpatia.

Aos meus colegas de trabalho, nomeadamente à Jani Silva, Nair Campos, Liliana e Carla Gabriel pelos conselhos, pelo apoio e principalmente pelos bons momentos.

Aos restantes membros do FP-ENAS/CEBIMED, aos professores, técnicos e auxiliares o meu muito obrigado pela simpatia e pela disponibilidade.

À Mariana Santos, porque a tua amizade é indispensável, assim como os teus conselhos, obrigada por toda a ajuda e por, apesar de seres a que está mais longe, estares sempre presente.

Ao Raphael Costa, pela enorme paciência, por me ajudares em tudo que podes, por estares ao meu lado em todos os momentos e porque o teu amor me ajudou a superar os piores momentos.

Aos meus pais, porque as palavras não são suficientes para vos agradecer por tudo que fizeram e fazem por mim, por serem um exemplo de humildade e determinação, porque sempre me apoiaram incondicionalmente e porque me dão força para nunca desistir. Agradeço do fundo do coração todo o esforço que fazem diariamente para me proporcionarem uma vida melhor.

Table of contents

List of figures

List of tables

Nomenclature

1,2-DHX: 1,2-Dihydroxyxanthone

AP-1: activator protein 1

APC: antigen-presenting cell

cdc2: cell division cycle protein 2 homolog

CDK: cyclin-dependent kinases

CHEK2 – checkpoint kinase 2

CTL or CD8+ T: cytotoxic T cell

CYP: cytochrome P450;

DMF: N.N-Dimetilformamida

DMSO: Dimethyl sulfoxide

DMXAA or Vadimezan: 5,6-dimethylxanthenone-4-acetic acid;

ELISA: Enzyme-Linked Immunosorbent Assay

NOS: nitric oxide synthases

FBS: fetal bovine serum

GI_{50}: half maximal growth-inhibitory concentrations

GST: glutathione-S-transferase

IC_{50}: half maximal inhibitory concentration

IL: interleukin;

INF-γ: interferon gamma

L-NAME: N-nitro-L-arginine methyl ester

LPS: lipopolysaccharides

M1: classic polarization of macrophages

M2: alternative polarization of macrophages;

MAPK: mitogen-activated protein kinase

MDM2: murine doble minute 2

MHC: major histocompatibility complex

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NF-κB: nuclear factor kappa B

NK: natural killer cell

NO: nitric oxide

$p21^{cip1}$: cyclin-dependent kinase inhibitor 1

p27: cyclin-dependent kinase inhibitor

p53: tumor protein p53

PBS: Phosphate buffered saline

PHA: phytohemaglutinin

PMA: phorbol-12-myristate-13-acetate;

SEM: standard error of the mean

SRB: sulphorrodamine B

TAM: tumor-associated macrophages

TGF: transforming growth factor

Th: T helper cell

TNF-α: tumour necrosis factor α

Treg: regulatory T cell

α-MG: Alpha-mangostin

Resumo

O cancro é uma das patologias mais agressivas e letais, permanecendo um desafio para investigadores e médicos, mesmo após décadas de investigação e investimento nesta área. A deteção tardia e as limitações das terapias atuais, associadas a quimio-resistência e efeitos adversos, são os principais problemas.

O melanoma é um tumor melanocítico altamente imunogénico e com elevada predisposição para metastizar. A sua incidência tem vindo a aumentar em todo o mundo e as taxas de mortalidade associadas a esta doença são muito elevadas. Deste modo, é necessário estabelecer terapias alternativas que superem as atuais limitações do tratamento, nomeadamente aumentando o espectro e a eficácia e diminuindo a toxicidade dos medicamentos usados atualmente. Uma boa estratégia para evitar a resistência tumoral exige moléculas com múltiplos alvos.

As xantonas são naturalmente obtidas em plantas de grande porte e também em microrganismos. Notáveis propriedades farmacêuticas foram descritas para estes compostos, principalmente o seu potencial anti-tumoral, que contribuiu para a comercialização de suplementos alimentares, para o isolamento e para a síntese de numerosos derivados xantónicos. No cancro, o efeito promovido por estes, compromete várias vias fisiológicas, interferindo com múltiplas características do cancro, incluindo a apoptose, ciclo celular, angiogénese, a inflamação e a vigilância imunitária, tornando-os um potencial candidato a fármaco.

O objetivo principal deste estudo foi avaliar os efeitos de duas xantonas, alfa-mangostina e 1,2-dihidroxixantona, no crescimento da linha celular humana de melanoma A375-C5 e a sua capacidade para modular o micro-ambiente imunológico dependente de macrófagos. Foi avaliada a capacidade dos compostos para interagir com os diferentes parâmetros imunológicos que podem interferir com o tratamento de tumores, incluindo a produção de óxido nítrico e a expressão de citocinas, como a interleucina1 beta e o fator de necrose tumoral alfa (características do fenótipo M1 em macrófagos) e a interleucina 10 e o fator transformador do crescimento beta 1 (características do fenótipo M2). A alfa-mangostina também foi testada em linfócitos, isto é, na proliferação celular e produção de citoquinas. Esta atividade foi previamente descrita para a 1,2-dihidroxixantona. Em todos os ensaios, foram usados sistemas celulares *in vitro*, maioritariamente de origem humana.

As xantonas causaram uma diminuição na produção de óxido nítrico e interferiram com a expressão de citocinas por macrófagos e / ou linfócitos estimulados. A alfa-mangostina inibiu a expressão da interleucina 1 beta e do fator transformador do crescimento beta 1, e estimulou o fator de necrose tumoral alfa. A 1,2-dihidroxixantona

inibiu a interleucina 1 beta e a interleucina 10, estimulou ainda o fator transformador do crescimento beta 1 e o fator de necrose tumoral alfa em macrófagos. A alfa-mangostina também interferiu com a proliferação de linfócitos e a expressão da interleucina 10 e do fator de necrose tumoral alfa por estas células.

Quanto ao impacto direto dessa modulação no crescimento de melanoma, os compostos apresentam uma melhoria de efeito citotóxico. No entanto, só no tratamento com 1,2-dihidroxixantona, esta melhoria pode ser associada com a modulação de macrófagos pela xantona, uma vez que o efeito da alfa-mangostina em doses baixas é semelhante aos macrófagos não tratados na linha celular em estudo.

Em conclusão, estes resultados permitiram inferir um impacto potencial da alfa-mangostina e da 1,2-dihidroxixantona no tratamento do melanoma, devido à sua atividade citotóxica e ao sugestivo efeito imunoterapêutico em tumores.

Abstract

Cancer is one of the most aggressive and lethal diseases, remaining a challenge for researchers and physicians, in spite of decades of research and investment in this area. Later detection and constrains of current therapies, associated with chemoresistance and toxic side effects, are the main problems.

Melanoma is a melanocytic tumor highly immunogenic and with a great predisposition to metastasize. Its incidence is increasing worldwide and the mortality rates associated to the disease are very high. Therefore, it is imperative to find therapeutic alternatives that overcome the actual limitations of the treatment, namely improving the spectrum and efficacy of actuation and decrease the toxicity of currently used drugs. A good strategy to avoid the tumor resistance needs a drug that targets multiple pathways.

Xanthones are naturally obtained from higher plants and microorganisms. Remarkable pharmacologic proprieties have been described for these compounds, mainly their antitumor potential, which contributed to their commercialization as dietary supplement, isolation and synthesis of numerous derivatives of xanthones. In cancer, their effect comprises several physiologic pathways, interfering with multiple hallmarks of cancer, including apoptosis, cell cycle, angiogenesis, inflammation and immune surveillance making them a prospective drug candidate.

The main gold of this study was the evaluation of the effects of two xanthones, alpha-mangostin and 1,2-dihydroxyxanthone, on A375-C5 melanoma cell growth and their capacity to modulate the immune macrophages-dependent microenvironment. The ability for the compounds to interact with different immunologic parameters that may interfere with tumor treatment were evaluated, including production of nitric oxide and expression of cytokines interleukin-1β and tumor necrosis factor-α (characteristics of M1 phenotype) and interleukin-10 and transforming growth factor-β1 (characteristic of M2 phenotype). alpha-Mangostin was also tested in lymphocytes, namely in cell proliferation and cytokines production. This activity was previously described for 1,2-dihydroxyxanthone. In all assays, *in vitro* cellular systems were used, mostly of human origin.

Xanthones caused a decrease in nitric oxide production and interfere with the expression of cytokines by stimulated macrophages and/or lymphocytes. Alpha-mangostin suppressed expression of interleukin-1β and transforming growth factor-β1, and stimulated tumor necrosis factor-α. 1,2-Dihydroxyxanthone inhibited interleukin-1β and interleukin-10, stimulated transforming growth factor-β1 and tumor necrosis factor-α in macrophages. Alpha-mangostin also interferes with lymphocytes proliferation and the expression of interleukin-10 and tumor necrosis factor-α by these cells. Concerning the direct impact of this modulation on melanoma growth, the compounds exhibit an

improvement of cytotoxic effect. However, only in 1,2-dihydroxyxanthone treatment, this improvement could be associated with modulation of macrophages, since the effect of alpha-mangostin at lower doses is similar to non-treated macrophages in the cell line in study.

In conclusion, these finds allowed to infer a prospective impact of alpha-mangostin and 1,2-dihydroxyxanthone in melanoma treatment, due to their cytotoxic activity and suggestive effect in cancer immunotherapy.

I. General Introduction

Xanthones, melanoma and immune system

Chemistry of xanthones: General considerations

Xanthones are biologically active tricyclic molecules characterized by a dibenzo-γ-pyrone nucleus or 9*H*-xanthen-9-one (**Figure 1**) (Pinto *et al.* 2005; Mazimba *et al.* 2013).

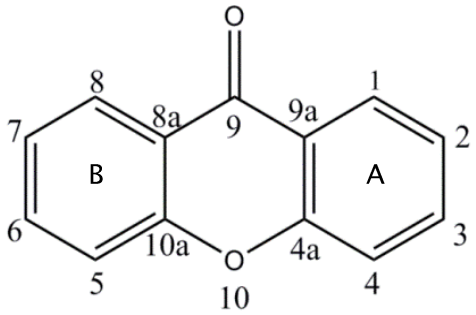


Figure 1 Xanthone basic skeleton

The diversity of xanthone derivatives is possible due to the variation of the nature and position of substituents on the A and B rings. According to that, natural xanthones may be categorized into: simple oxygenated, glycosylated, prenylated and their derivatives (xanthone dimers, xanthonolignoids, and miscellaneous). On the other hand, the synthetic xanthones can have simple groups such as hydroxyl, methoxyl, methyl, carboxyl, as well as more complex substituents such as epoxide, azole, methylidenebutyrolactone, aminoalcohol, sulfamoyl, methylthiocarboxylic acid, and dihydropyridine in their scaffold (Pinto *et al.* 2005).

Natural xanthones of higher plants mainly occur in two families, Guttiferae and Gentianaceae, and can also be found in microorganisms as fungi and lichens (Vieira and Kijjoa 2005; Mazimba *et al.* 2013). The majority of these compounds was obtained from *Garcinia mangostana* Linn, being the most abundant and frequently studied, the α-mangostin, β-mangostin, γ-mangostin, garcinone E and gartanin (**Figure 2**) (Shan *et al.* 2011).

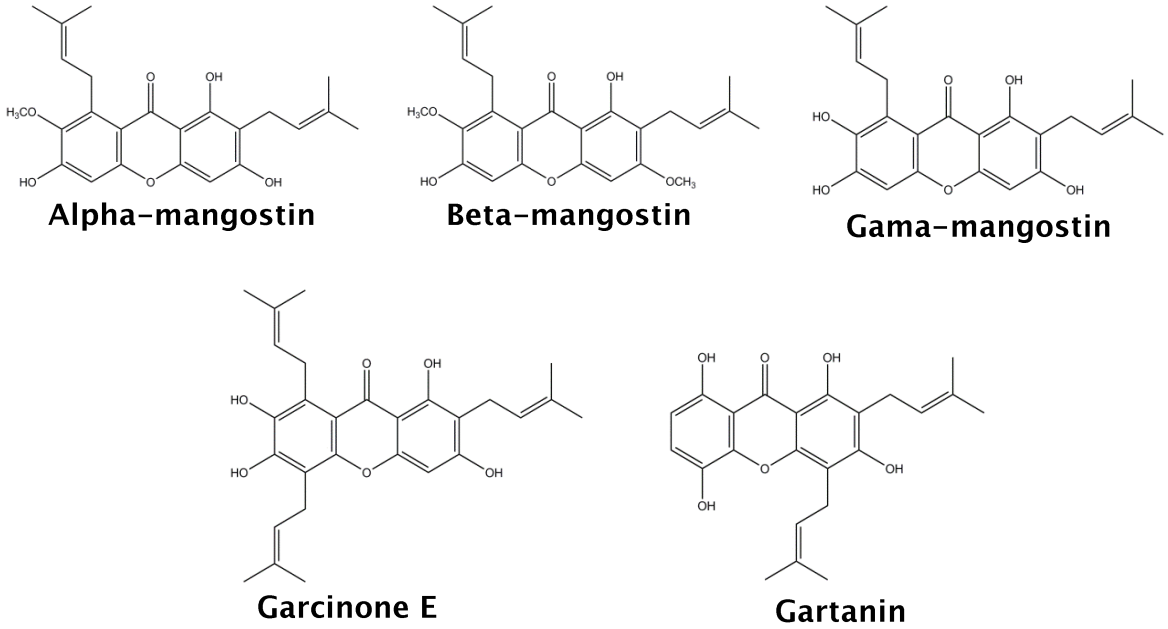


Figure 2: Structure of the main xanthones from mangosteen

Xanthones have shown remarkable biological/pharmacological activities linked with their tricyclic scaffold, depending on the nature and/or position of the diverse constituents (Mazimba *et al.* 2013). As xanthones from natural origin are quite limited in type and position of the substituents due to the biosynthetic pathways, the syntheses of new xanthones can attempt to alter or improve their activity by having different nature and positions of the substituents on the nucleus of these compounds (Pedro *et al.* 2002).

Biological activities of xanthones

In the last decade, the interest for natural or derivative xanthones has been growing as readily confirmed by the increased numbers of scientific reports (Gutierrez-Orozco and Failla 2013). These have allowed to find a great variety of biological/pharmacological activities associated with xanthone derivatives including analgesic (Bianco *et al.* 1989; Garrido *et al.* 2001; Cui *et al.* 2010), antioxidant (Madan *et al.* 2002; Jung *et al.* 2006), anti-inflammatory (Lin *et al.* 1996; Madan *et al.* 2002; Teixeira *et al.* 2005; Chen *et al.* 2008), antitumor (Pinto *et al.* 2005; Pedraza-Chaverri *et al.* 2008; Shan *et al.* 2011; Gutierrez-Orozco and Failla 2013), antiallergic (Pfister *et al.* 1972; Nakatani *et al.* 2002), antimicrobial (Pinto *et al.* 2005; Pedraza-Chaverri *et al.* 2008), neuroprotective (Li and Ohizumi 2004; Weecharangsan *et al.* 2006) and immunomodulatory (Makare *et al.* 2001; Tang *et al.* 2009).

In spite of all promising proprieties in the improvement of treatment of a number of pathologies as cancer (Pinto *et al.* 2005; Shan *et al.* 2011), diabetes (Ichiki *et al.* 1998;

Bumrungpert *et al.* 2009), cardiac (Jiang *et al.* 2004; Devi Sampath and Vijayaraghavan 2007), psychiatric (Chairungsrilerd *et al.* 1996), autoimmune (Madan *et al.* 2002; Yusuf-Makagiansar *et al.* 2002; Leiro *et al.* 2004) and neurodegenerative pathologies (Weecharangsan *et al.* 2006; El-Seedi *et al.* 2010), it is important to note that the majority of the compounds were only evaluated in cell lines or animal models. According to that the safety and efficacy of these products cannot be completely assure.

Vadimezan (5,6-dimethylxanthenone-4-acetic acid, DMXAA), a promising anticancer xanthone that presented important proprieties as vascular disrupting-agent is an example of the need of appropriated clinical trial. This compound was tested until phase II trial suggesting potential application in combination with paclitaxel and carboplatin for non-small-cell lung cancer (McKeage *et al.* 2009). Although, the phase III revealed a lack of utility to human use against this cancer due to a specie-specific role (Baguley and Ching; Lara *et al.* 2011).

Antitumor activity

Among all physiological activities mediated by xanthone compounds, the antitumor capacity seemed to be quite remarkable since they exert their inhibitory effect in a significant range of tumors. This activity was demonstrated *in vitro* and/or *in vivo* on breast (Pedro *et al.* 2002; Moongkarndi *et al.* 2004; García-Rivera *et al.* 2011), colorectal (Gobbi *et al.* 2002; Nakagawa *et al.* 2007; Watanapokasin *et al.* 2010), prostate (Johnson *et al.* 2012), colon (Chitchumroonchokchai *et al.* 2013), lung (Kostakis *et al.* 2002; Suksamrarn *et al.* 2006; Rajendran *et al.* 2008), glioma (Chao *et al.* 2011), hepatoma (Ho *et al.* 2002; Zou *et al.* 2004), leukemia (Kostakis *et al.* 2002; Seo *et al.* 2002; Matsumoto *et al.* 2003; Yao *et al.* 2010) and melanoma (Joseph *et al.* 1999; Pedro *et al.* 2002; Wang *et al.* 2011), among others (Pinto *et al.* 2005; Gutierrez-Orozco and Failla 2013). Analyze analyse

The chemotherapeutic and chemopreventive potential owing their inhibitory effect on every steps in the process of tumorigenesis (initiation, promotion, and progression) and on multiple signaling targets (Sun *et al.* 2002; Liu *et al.* 2013). Indeed, these compounds are able to modulate a considerable number of hallmarks of cancer by induce cell cycle arrest, suppression of tumor cell proliferation, induction of apoptosis, differentiation, reduction of inflammation, and inhibition of adhesion, invasion, and metastasis (Pinto *et al.* 2005; Akao *et al.* 2008; Pedraza-Chaverri *et al.* 2008; Shan *et al.* 2011; Gutierrez-Orozco and Failla 2013).

The induction of apoptosis may be possible, among other factors, due activation of caspase cascade and disruption of mitochondrial membrane and consequent release of cytochrome c (Matsumoto *et al.* 2004). Antiproliferative effects of xanthones were demonstrated as result of cell cycle arrest at G1 and S phases (Matsumoto *et al.* 2005).

The inhibition of metastatic process is likely associated to inhibition of matrix metalloproteinase (MMPs; particularly MMP-2 and MMP-9) and u-PA (urokinase - plasminogen activator) expression by JNK1/2 (Jun N-terminal kinase), NF-κB (nuclear factor kappa-B) and AP-1 (activator protein 1) activity suppression (Hung *et al.* 2009).

Therefore, xanthones could be agents of an emerging antitumor therapy capable of alter multiple signaling targets, affect various traits of cancer and consequently prevent adaptive resistance (Hanahan and Weinberg 2011; Liu *et al.* 2013).

Anti-inflammatory activity

There is a range of evidences from the involvement of inflammatory pathways in tumorigenesis (Kundu and Surh 2008). Xanthones have demonstrated both, antitumor and anti-inflammatory potential.

Several xanthone derivatives, mainly the natural ones, attenuated the expression of inflammatory mediators as TNF-α (tumor necrosis factor-α) and interleukins 6 (IL-6) in cell lines of macrophages and adipocytes and decreased the activation of signaling pathways including IL-1, mitogen-activated protein kinase (MEK), Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), signal transducer and activator of transcription 1 (STAT-1), NF-κB, AP-1 in these cells (Kumar *et al.* 2003; Leiro *et al.* 2004; Bumrungpert and Kalpravidh 2010; Liu *et al.* 2012). Immunomodulatory effects were also observed in murine models (Jang *et al.* 2012).

Reduction of inducible NO synthase (iNOS) mRNA was reported for several xanthones in murine macrophages cell lines (Garrido *et al.* 2004; Teixeira *et al.* 2005; Chen *et al.* 2008; Tewtrakul *et al.* 2009) or murine models (Leiro *et al.* 2003). Decreased levels of cyclooxygenase-2 (COX-2) were also associated with activity of xanthones (Leiro *et al.* 2004; Chen *et al.* 2008; Tewtrakul *et al.* 2009).

This represents a small part of all the reported anti-inflammatory effects of xanthones, many others could be added to those as demonstrated in some published reviews (Pinto *et al.* 2005; Shan *et al.* 2011; Gutierrez-Orozco *et al.* 2013).

Melanoma

Melanoma is a malignancy of melanocytes or their precursors, the melanoblasts (Asnaghi *et al.* 2012). This is primarily located in the skin, but can also be found in ears, gastrointestinal tract, eyes, oral and genital mucosa and leptomeninges (McCourt *et al.* 2014). The main risk factor associated to this cancer is the excess of sun exposition as repeatedly referred in several prevention campaigns. In spite of this attempt to control

emerging cases, its incidence rate is still rising worldwide at highest levels than other tumors (Maio 2012; Liu *et al.* 2014).

Melanocytic tumors presented a highly predisposition to metastasize, however, when early detected frequently means a successful treatment and increasing of survival. Contrariwise, metastatic tumors represent poor prognostic with high associated mortality (Korn *et al.* 2008; Gast *et al.* 2011).

Melanoma tumorigenesis requires a multistep process, however, a great variety of evidences referred the crucial involvement of immune system in tumor progression (Hussein 2004). The character highly immunogenic of melanocytic tumors and the influence of host immune response and microenvironment inflammatory cells in cancer growth were verified (Hussein 2004; Dranoff 2009; Chen *et al.* 2011). Indeed, primary melanomas undergo spontaneous regression much more frequently than any other cancer that may be a sign of immunosurveillance or, by other way, it may be due melanoma are easier to visualize (Printz 2001; Kalialis *et al.* 2009). Nevertheless, other evidences corroborate the involvement of immune system, namely the relatively amount of tumor-infiltrating lymphocytes in melanoma microenvironment compared with other cancers and associated with favorable prognostic (Maio 2012; Kushnir and Merimsky 2013) and the appearance of autoimmune condition as vitiligo in melanoma patients or patients treated with immunotherapy which normally means a better disease outcome (Le Gal *et al.* 2001; Phan *et al.* 2001; Boasberg *et al.* 2006).

In more advanced stages, this cancer became capable to avoid immunosurveillance (Reiman *et al.* 2007; Speeckaert *et al.* 2011) and its progression and metastatic potential may be supported by immune cells present in tumor microenvironment, as the tumor-associated macrophages (TAMs) since they are the most abundant leucocytes in melanoma and represent a poor prognostic (Brocker *et al.* 1988; Bernengo *et al.* 2000; Makitie *et al.* 2001; Varney *et al.* 2005; Porta *et al.* 2007; Solinas *et al.* 2009; Mantovani and Sica 2010; Qian and Pollard 2010).

Immune System

Immune system is a complex network of cells, tissues and organ that plays an important role in defense against multiple microorganisms and toxins and it is essential to organism homeostasis by respond not only to exogenous, but also to endogenous signals (Matzinger 2002). As is known, this system could be divided in two components, the innate and adaptive immunity.

Innate immunity is the immediate response to a “danger” and comprises a variety of cells, including dendritic cells (DC), macrophages, neutrophils and natural killer (NK) cells (Vesely *et al.* 2011; Lakshmi Narendra *et al.* 2013).

Adaptive immunity is antigen specific due to somatic rearrangement on genes that codify each receptor of lymphocytes, i.e. the T cell receptor (TCR) for T lymphocytes and immunoglobin for B lymphocytes. Beyond T and B lymphocytes, adaptive immune system comprises humoral mediators including cytokines and antibodies (Vesely *et al.* 2011; Lakshmi Narendra *et al.* 2013).

Macrophages

Macrophages are essential cells of innate immune system that play an important role in host defense and tissue homeostasis maintenance (Gordon and Martinez 2010). They derived from circulating monocytes originates from monocytic precursors of bone marrow. Monocytes are attracted to target tissues where differentiate into mature macrophages (Murray and Wynn 2011; Davies *et al.* 2013).

Macrophages are heterogeneous cells able to polarize in different subtypes among the classic phenotype (M1) and the alternatives (M2-like), according to the received stimuli which influence their immune response (**Figure 3**) (Mantovani *et al.* 2002; Martinez *et al.* 2009). The M1 phenotype is triggered by Th1 (T-helper 1 lymphocyte) cytokine interferon-γ (IFN-γ), bacteria constituents (like LPS) or Toll-like receptors (TLRs) agonists. Once activated, M1 macrophages produce pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-23, great quantities of MHC (major histocompatibility complex) class I and II (essential for antigen presentation), nitric oxide (NO) and pro-inflammatory chemokines. They promote the recruitment of Th1 cells, CD8+ CTL (cytotoxic T cells) and NK cells leading to an inflammatory response and antitumor immunity. (Mantovani *et al.* 2002; Mantovani *et al.* 2004; Fairweather and Cihakova 2009; Hao *et al.* 2012; Sica and Mantovani 2012).

Conversely, M2 macrophages are activated by Th2 cytokines, including IL-4 and IL-13, or only by IL-10. They express high levels of IL-10 and TGF-β and immunosuppressive chemokines. M2-like phenotype favors the recruitment and development of Treg (regulatory T cells) and Th2, leading to a response that supports tumor growth through immunosuppression (Coffelt *et al.* 2009; Martinez *et al.* 2009; Siveen and Kuttan 2009; Hao *et al.* 2012; Sica and Mantovani 2012). However, is difficult to establish a linear effect of M1 and M2 macrophages in immune system.

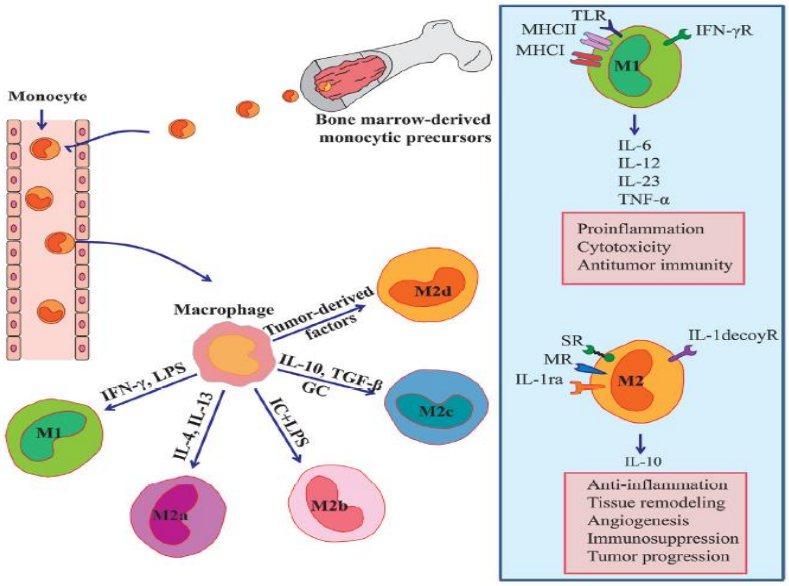


Figure 3: Polarization of macrophages and corresponding functions. Legend: GC indicate glucocorticoid; IC, immune complex; IL-1ra, IL-1 receptor antagonist; LPS, lipopolysaccharide; MR, mannose receptor; SR, scavenger receptor - adapted from (Chanmee *et al.* 2014).

T lymphocytes

T lymphocytes or T cells derived from lymphoid precursors and develop in the thymus. They express specific receptors (TCRs) capable to recognize peptides (antigens) presented by APCs (antigen-presenting cells, namely dendritic cells) and direct or indirectly by macrophages. T cells may be classified into cytotoxic T lymphocytes (CD8+ CTL) when recognize peptides presented by MHC class I or T helper cells (CD4+ Th) if they detect antigens from MHC class II (Fauci *et al.* 2009; Lakshmi Narendra *et al.* 2013).

Naïve CD8+ T could proliferate and differentiate into effectors (or CTLs) and memory CD8+ T cells after appropriate activation signals. CTLs produce cytokines (as INF-γ) and effector molecules (perforin and granzyme-B) with cytotoxic effect on the targets including infected or cancer cells (Lakshmi Narendra *et al.* 2013).

CD4+ T cells are a much heterogeneous group: they can be subdivided into Th1 (expressing IFN-γ, TNF-α and IL-2), Th2 (expressing IL-4-, IL-5, IL-10 and IL-13), Th17 (producing IL-17), T follicular helper cells (TFH), and Treg depending of stimuli (Munk and Emoto 1995; Wilson *et al.* 2009; Zhou *et al.* 2009).

Th1 collaborate with CTLs and regulate the duration and magnitude of pro-inflammatory or anti-tumor CTLs actions (Romagnani *et al.* 1997).

In contrast with Th1, Th2 induce T cells anergy, suppressing CTLs response and promoting protumor humoral immune response (Parker 1993; Lakshmi Narendra *et al.* 2013).

Regulatory T cells (Treg) may be separated into natutal (nTreg) and inducible (iTreg or Tr1). Natural Treg mediate immunossupressive via cell contact-dependent mechanisms (as granzyme-B/perforin or Fas/Fas ligand pathways) and maintain the immune tolerance (Raimondi *et al.* 2007). Tr1 is induced by microenvironment signals as antigens, IL-2, TGF-β and IL-10, and exert an immunosuppressive effect by produce anti-inflammatory cytokines (Roncarolo *et al.* 2001; Roncarolo *et al.* 2006; Bergmann *et al.* 2007).

Nitric Oxide

Nitric oxide (NO) is an endogenous gas produced by nitric oxide synthases (NOS) by L-arginine. It is an important cellular signaling molecule involved either in physiologic (including maintenance of blood pressure, neuronal mediation and inflammation) or pathological process (as vascular shock, stroke, diabetes, arthritis, chronic inflammation and cancer) (Gross and Wolin 1995; Kuo and Schroeder 1995; Hou *et al.* 1999). In mammalians, the main isoforms of the NOS enzyme are the endothelial (eNOS or NOS3), neuronal (nNOS or NOS1) and inducible (iNOS or NOS2). NOS1 and NOS3 are expressed constitutively in neuronal and endothelial cells, respectively, depending on calcium concentration. NOS2 is an inducible calcium-independent isoform expressed after immunologic stimuli in, theoretically, all cells (Moncada *et al.* 1991; Gross and Wolin 1995; U. 2012).

In cancer, NO effect seemed to be biphasic. Below a critical concentration of NO, it causes DNA mutations (Wink *et al.* 1998), inhibits apoptosis (Choi *et al.* 2002), promotes angiogenesis (Ziche and Morbidelli 2000), limits immune response against cancer (Wink *et al.* 1991) and promotes metastasis (Lala and Orucevic 1998). When it exceeds the critical concentration, NO induce apoptosis and suppress the growth of the tumor (Choudhari *et al.* 2013).

Cytokines

Cytokines are small proteins essentials to the interaction and communications between cells. They interact in a complex pathway in an autocrine, paracrine or endocrine manner with synergic or antagonist outcome, since a single cytokine may act on several different cell types (**Figure 4**) (Zhang and An 2007).

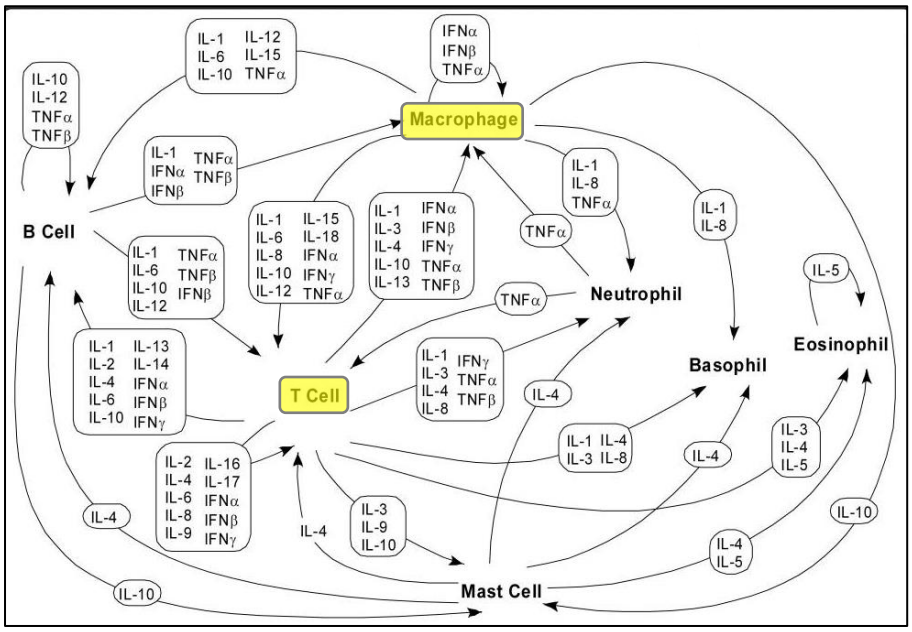


Figure 4 Cytokine network in immune system – adapted from (Zhang and An 2007)

Monokines, interleukins and lymphokines are different types of cytokines. They are produced by a variety of immune or endothelial cells; however helper T cells (Th) and macrophages are the predominant producers (Zhang and An 2007).

In spite of their importance in several physiologic processes, they have been also associated to pathologic condition. Tumor cell and cells from tumor microenvironment, for example, showed aberrant production of cytokine (Kurzrock 2001; Jin *et al.* 2004).

Cytokines are commonly divided in pro-inflammatory and anti-inflammatory. Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are pro-inflammatory chemokines frequently associated either to immunosurveillance against cancer, or tumor progression. Anti-inflammatory cytokines, as transforming growth factor- β (TGF-β) and IL-10, also show complex effects on tumor development.

IL-1β is only produced after stimulation by inflammatory signals. It has been associated with all steps of malignancy (carcinogenesis, progression, invasion and metastasis) (Apte and Voronov 2008) and was related with metastasis promotion in melanoma models *in vivo* (Giavazzi *et al.* 1990; Meyer *et al.* 2011). In contrast, this interleukin is highly produced by M1 macrophages, important promoters of immune response against

malignant cells, which means that IL-1β may facilitate immunosurveilance, limiting tumor growth and progression (Fairweather and Cihakova 2009).

TNF-α can induce different signaling pathways, including a pro-apoptotic and an anti-apoptotic, among others (Chen and Goeddel 2002). According to that, its effect is a double-edge sword in carcinogenesis. Higher concentration of this cytokine can induce an antitumor response (Wiemann and Starnes 1994; Herman *et al.* 2013). In agreement, inhibitors of TNF appear to increase the risk of skin cancer, including melanoma (Mariette *et al.* 2011; Kouklakis *et al.* 2013). In contrast, low levels of it can induce tumor phenotypes by promote reactive oxygen/nitrogen species generation, causing DNA damage and promoting tumorigenesis (Woo *et al.* 2000; Hussain *et al.* 2003; Balkwill 2006).

TGF-β presents immunosuppressive and anti-inflammatory properties, involved in multiple physiologic pathways including embryogenesis, proliferation and differentiation (Santibanez *et al.* 2011). Their role in cancer is controversial. In early stages of cancer it promotes cell cycle arrest and apoptosis, acting as tumor suppressor. In later stages TGF-β stimulated the invasion and metastasis, inducing epithelial-mesenchymal transition (Akhurst and Derynck 2001; Morrison *et al.* 2013). In melanoma, TGF-β isoforms (TGF-β1/2/3) are highly expressed and increase in parallel with tumor progression (Krasagakis *et al.* 1998; Javelaud *et al.* 2008). According to that, this cytokine is commonly produced by M2-like macrophages leads to a Th2 response, i.e., promotes an immunosuppressive microenvironment that allows the tumor immune escape (Coffelt *et al.* 2009; Martinez *et al.* 2009; Siveen and Kuttan 2009; Hao *et al.* 2012; Sica and Mantovani 2012).

IL-10 is produced by almost all immune cells (as T and B cell, macrophages and monocytes, mast cells, granulocytes, dendritic cells and keratinocytes) (Sabat *et al.* 2010; Costa *et al.* 2013) and can also be produced by tumor cells (Gastl *et al.* 1993). In cancer, its effect is, once again, paradoxical. IL-10 can exert antitumor activity in glioma, melanomas, breast and ovarian carcinomas (Lin and Karin 2007) may be through downregulation of MHC I and consequent induction of NK-mediated tumor cell lysis (Kundu and Fulton 1997). However, IL-10 can allow the immune escape by tumor and reduce antigen presentation, cell maturation, differentiation and apoptosis (Zeng *et al.* 2010; Hamidullah *et al.* 2012).

Tumor immunology

In case of malignant transformation, characteristic mutations of cancer produce tumor-associated antigens (TAAs) that may allow immune activation and tumor control (Spurrell and Lockley 2014).

However, the persistence of tumors implies immune scape by several mechanisms. Same tumors lose essential functions to immune activation as the failure in expression of MHC molecules (responsible to antigen presentation and consequent T cell activation). Others express immunosuppressive cytokines or create physical barriers to avoid immunosurveillance (Spurrell and Lockley 2014).

From all inflammatory cells, macrophages or tumor-associated macrophages (TAMs) seemed to have the most important functions in malignancies (Chen *et al.* 2011). In fact, during tumor development macrophages have mostly M1 phenotypes and promote an immune response against cancer, however, in later-stages of tumor, TAMs frequently exhibit M2-like phenotypes (Sica and Mantovani 2012). M2-like TAMs represent a worst prognostic, since they favor tumor growth and survival through induction of angiogenesis and suppression of cytotoxic activity of T lymphocytes (Tsutsui *et al.* 2005; Zijlmans *et al.* 2006; Mantovani and Sica 2010).

Figure 5 represents the interaction between innate and adaptive immunity and consequent effects on tumors. It reinforces the controversial effect of immune system, i.e. a set of immune cell (as M1 macrophages, NK, CTLs and Th1 cells) and specifics cytokines detect and control tumor progression, while other cells (like M2-macrophages, Th2 and Tregs) and derived cytokines, frequently present at later stages of cancer, contribute to their progression and immune scape.

Nevertheless, the role of immune system in cancer is not so linear because, this is influenced by the tumor microenvironment. Some immune cells are heterogeneous and present high plasticity, altering their phenotypes in response to cancer which explains the frequent inconsistent effects verified. In fact, the protumor or antitumor effects of each immune cell depend on cytokines signaling present in tumor microenvironment and it will determine their mode of differentiation (Lakshmi Narendra *et al.* 2013).

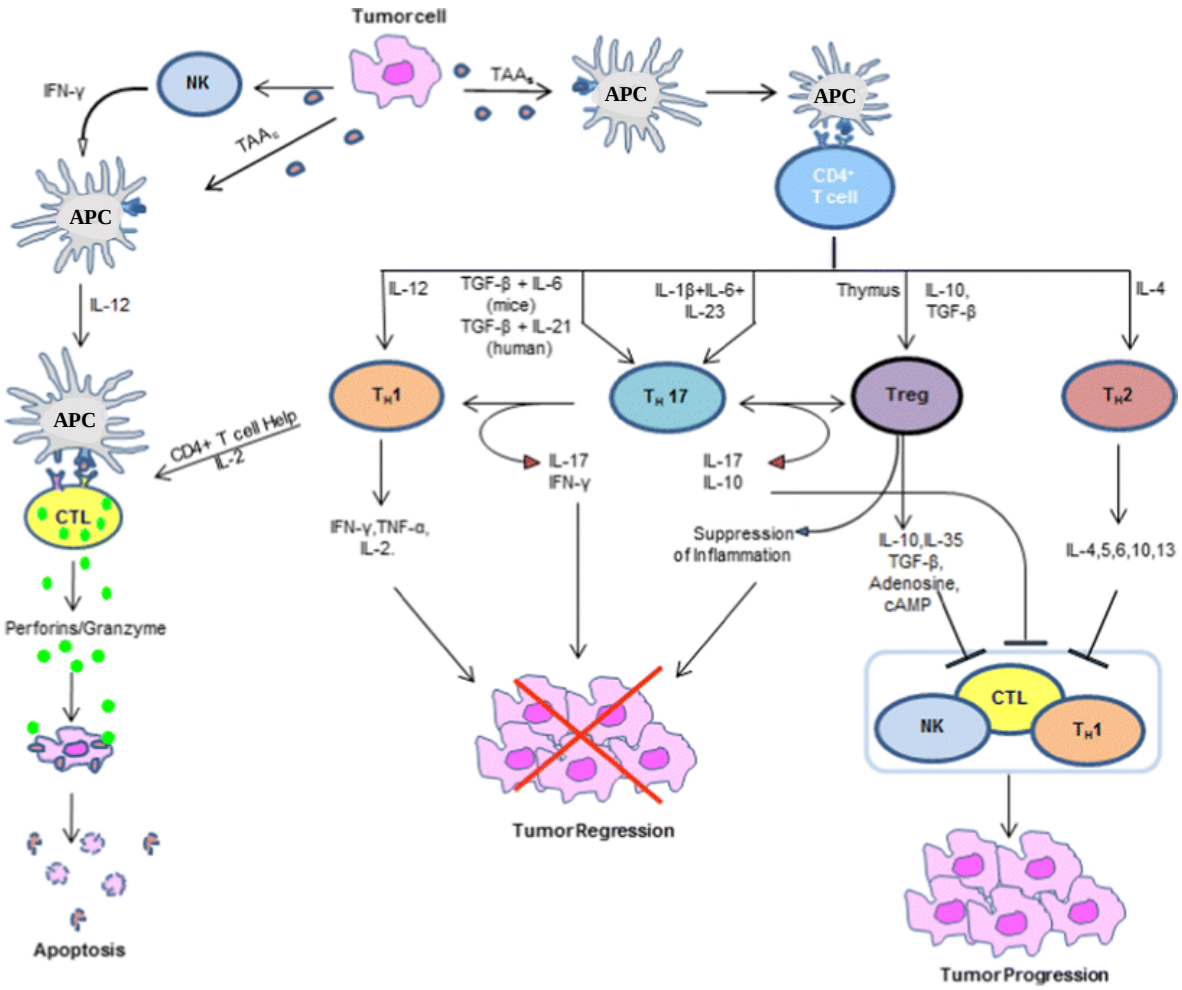


Figure 5: Schematic presentation of the role of immune system in cancer. Legend: APC indicates antigen - presenting cell; CTL, cytotoxic T lymphocyte or CD8+ T cell; NK, natural killer cell; Th, T helper cell; Treg, regulatory T cell and TAA, tumor-associated antigens - adapted from (Lakshmi Narendra *et al.* 2013).

References

Akao Y, Nakagawa Y, Iinuma M *et al* (2008) Anti-cancer effects of xanthones from pericarps of mangosteen. Int J Mol Sci 9: 355-70.

Akhurst RJ & Derynck R (2001) TGF-β signaling in cancer – a double-edged sword. Trends in Cell Biology 11: S44-S51.

Apte RN & Voronov E (2008) Is interleukin-1 a good or bad 'guy' in tumor immunobiology and immunotherapy? Immunological Reviews 222: 222-241.

Asnaghi L, Ebrahimi KB, Schreck KC *et al* (2012) Notch signaling promotes growth and invasion in uveal melanoma. Clin Cancer Res 18: 654-65.

Baguley BC & Ching L-M DMXAA: An antivascular agent with multiple host responses. International Journal of Radiation Oncology • Biology • Physics 54: 1503-1511.

Balkwill F (2006) TNF-alpha in promotion and progression of cancer. Cancer Metastasis Rev 25: 409-16.

Bergmann C, Strauss L, Zeidler R *et al* (2007) Expansion of human T regulatory type 1 cells in the microenvironment of cyclooxygenase 2 overexpressing head and neck squamous cell carcinoma. Cancer Res 67: 8865-73.

Bernengo MG, Quaglino P, Cappello N *et al* (2000) Macrophage-mediated immunostimulation modulates therapeutic efficacy of interleukin-2 based chemoimmunotherapy in advanced metastatic melanoma patients. Melanoma Res 10: 55-65.

Bianco A, Passacantilli P, Righi G *et al* (1989) Synthesis of 2-hydroxyacetyl-7-acetyl-xanthone, a new xanthone derivative endowed with antianaphylactic, analgesic, and antiinflammatory activities. Farmaco 44: 547-54.

Boasberg PD, Hoon DS, Piro LD *et al* (2006) Enhanced survival associated with vitiligo expression during maintenance biotherapy for metastatic melanoma. J Invest Dermatol 126: 2658-63.

Brocker EB, Zwadlo G, Holzmann B *et al* (1988) Inflammatory cell infiltrates in human melanoma at different stages of tumor progression. Int J Cancer 41: 562-7.

Bumrungpert A, Kalpravidh R, Chitchumroonchokchai C *et al* (2009) Xanthones from mangosteen prevent lipopolysaccharide-mediated inflammation and insulin resistance in primary cultures of human adipocytes. The Journal of Nutrition: 1185-1191.

Bumrungpert A & Kalpravidh RW (2010) Xanthones from mangosteen inhibit inflammation in human macrophages and in human adipocytes exposed to macrophage-conditioned media. The Journal of …: 1-6.

Chairungsrilerd N, Furukawa K, Ohta T *et al* (1996) Histaminergic and serotonergic receptor blocking substances from the medicinal plant Garcinia mangostana. Planta Med 62: 471-2.

Chanmee T, Ontong P, Konno K *et al* (2014) Tumor-associated macrophages as major players in the tumor microenvironment. Cancers (Basel) 6: 1670-90.

Chao AC, Hsu YL, Liu CK *et al* (2011) alpha-Mangostin, a dietary xanthone, induces autophagic cell death by activating the AMP-activated protein kinase pathway in glioblastoma cells. J Agric Food Chem 59: 2086-96.

Chen G & Goeddel DV (2002) TNF-R1 signaling: a beautiful pathway. Science 296: 1634-5.

Chen LG, Yang LL & Wang CC (2008) Anti-inflammatory activity of mangostins from Garcinia mangostana. Food Chem Toxicol 46: 688-93.

Chen P, Huang Y, Bong R *et al* (2011) Tumor-associated macrophages promote angiogenesis and melanoma growth via adrenomedullin in a paracrine and autocrine manner. Clinical cancer research : an official journal of the American Association for Cancer Research 17: 7230-9.

Chitchumroonchokchai C, Thomas-Ahner JM, Li J *et al* (2013) Anti-tumorigenicity of dietary alpha-mangostin in an HT-29 colon cell xenograft model and the tissue distribution of xanthones and their phase II metabolites. Mol Nutr Food Res 57: 203-11.

Choi BM, Pae HO, Jang SI *et al* (2002) Nitric oxide as a pro-apoptotic as well as anti-apoptotic modulator. J Biochem Mol Biol 35: 116-26.

Choudhari SK, Chaudhary M, Bagde S *et al* (2013) Nitric oxide and cancer: a review. World J Surg Oncol 11: 118.

Coffelt SB, Hughes R & Lewis CE (2009) Tumor-associated macrophages: effectors of angiogenesis and tumor progression. Biochim Biophys Acta 1796: 11-8.

Costa NL, Valadares MC, Souza PP *et al* (2013) Tumor-associated macrophages and the profile of inflammatory cytokines in oral squamous cell carcinoma. Oral Oncol 49: 216-23.

Cui J, Hu W, Cai Z *et al* (2010) New medicinal properties of mangostins: analgesic activity and pharmacological characterization of active ingredients from the fruit hull of Garcinia mangostana L. Pharmacol Biochem Behav 95: 166-72.

Davies LC, Jenkins SJ, Allen JE *et al* (2013) Tissue-resident macrophages. Nat Immunol 14: 986-95.

Devi Sampath P & Vijayaraghavan K (2007) Cardioprotective effect of alpha-mangostin, a xanthone derivative from mangosteen on tissue defense system against isoproterenol-induced myocardial infarction in rats. J Biochem Mol Toxicol 21: 336-9.

Dranoff G (2009) Targets of protective tumor immunity. Ann N Y Acad Sci 1174: 74-80.

El-Seedi HR, El-Barbary MA, El-Ghorab DM *et al* (2010) Recent insights into the biosynthesis and biological activities of natural xanthones. Curr Med Chem 17: 854-901.

Fairweather D & Cihakova D (2009) Alternatively activated macrophages in infection and autoimmunity. J Autoimmun 33: 222-30.

Fauci A, Braunwald E, Kasper D *et al* (2009) Harrison's Manual of Medicine, 17th Edition, McGraw-Hill Education.

García-Rivera D, Delgado R, Bougarne N *et al* (2011) Gallic acid indanone and mangiferin xanthone are strong determinants of immunosuppressive anti-tumour effects of Mangifera indica L. bark in MDA-MB231 breast cancer cells. Cancer Letters 305: 21-31.

Garrido G, Delgado R, Lemus Y *et al* (2004) Protection against septic shock and suppression of tumor necrosis factor alpha and nitric oxide production on macrophages and microglia by a standard aqueous extract of Mangifera indica L. (VIMANG). Role of mangiferin isolated from the extract. Pharmacol Res 50: 165-72.

Garrido G, Gonzalez D, Delporte C *et al* (2001) Analgesic and anti-inflammatory effects of Mangifera indica L. extract (Vimang). Phytother Res 15: 18-21.

Gast A, Bermejo JL, Claus R *et al* (2011) Association of inherited variation in Toll-like receptor genes with malignant melanoma susceptibility and survival. PLoS One 6: e24370.

Gastl GA, Abrams JS, Nanus DM *et al* (1993) Interleukin-10 production by human carcinoma cell lines and its relationship to interleukin-6 expression. International Journal of Cancer 55: 96-101.

Giavazzi R, Garofalo A, Bani MR *et al* (1990) Interleukin 1-induced augmentation of experimental metastases from a human melanoma in nude mice. Cancer Res 50: 4771-5.

Gobbi S, Rampa A, Bisi A *et al* (2002) Synthesis and antitumor activity of new derivatives of xanthen-9-one-4-acetic acid. J Med Chem 45: 4931-9.

Gordon S & Martinez FO (2010) Alternative activation of macrophages: mechanism and functions. Immunity 32: 593-604.

Gross SS & Wolin MS (1995) Nitric oxide: pathophysiological mechanisms. Annu Rev Physiol 57: 737-69.

Gutierrez-Orozco F, Chitchumroonchokchai C, Lesinski GB *et al* (2013) alpha-Mangostin: anti-inflammatory activity and metabolism by human cells. J Agric Food Chem 61: 3891-900.

Gutierrez-Orozco F & Failla ML (2013) Biological activities and bioavailability of mangosteen xanthones: a critical review of the current evidence. Nutrients 5: 3163-83.

Hamidullah, Changkija B & Konwar R (2012) Role of interleukin-10 in breast cancer. Breast Cancer Res Treat 133: 11-21.

Hanahan D & Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144: 646-74.

Hao N-B, Lü M-H, Fan Y-H *et al* (2012) Macrophages in Tumor Microenvironments and the Progression of Tumors. Clinical and Developmental Immunology 2012: 11.

Herman JM, Wild AT, Wang H *et al* (2013) Randomized phase III multi-institutional study of TNFerade biologic with fluorouracil and radiotherapy for locally advanced pancreatic cancer: final results. J Clin Oncol 31: 886-94.

Ho CK, Huang YL & Chen CC (2002) Garcinone E, a xanthone derivative, has potent cytotoxic effect against hepatocellular carcinoma cell lines. Planta Medica 68: 975-979.

Hou YC, Janczuk A & Wang PG (1999) Current trends in the development of nitric oxide donors. Curr Pharm Des 5: 417-41.

Hung SH, Shen KH, Wu CH *et al* (2009) Alpha-mangostin suppresses PC-3 human prostate carcinoma cell metastasis by inhibiting matrix metalloproteinase-2/9 and urokinase-plasminogen expression through the JNK signaling pathway. J Agric Food Chem 57: 1291-8.

Hussain SP, Hofseth LJ & Harris CC (2003) Radical causes of cancer. Nat Rev Cancer 3: 276-85.

Hussein MR (2004) Genetic pathways to melanoma tumorigenesis. J Clin Pathol 57: 797-801.

Ichiki H, Miura T, Kubo M *et al* (1998) New antidiabetic compounds, mangiferin and its glucoside. Biol Pharm Bull 21: 1389-90.

Jang HY, Kwon OK, Oh SR *et al* (2012) Mangosteen xanthones mitigate ovalbumin-induced airway inflammation in a mouse model of asthma. Food Chem Toxicol 50: 4042-50.

Javelaud D, Alexaki VI & Mauviel A (2008) Transforming growth factor-beta in cutaneous melanoma. Pigment Cell Melanoma Res 21: 123-32.

Jiang DJ, Dai Z & Li YJ (2004) Pharmacological effects of xanthones as cardiovascular protective agents. Cardiovasc Drug Rev 22: 91-102.

Jin P, Panelli MC, Marincola FM *et al* (2004) Cytokine polymorphism and its possible impact on cancer. Immunol Res 30: 181-90.

Johnson JJ, Petiwala SM, Syed DN *et al* (2012) α-Mangostin, a xanthone from mangosteen fruit, promotes cell cycle arrest in prostate cancer and decreases xenograft tumor growth. Carcinogenesis 33: 413-419.

Joseph WR, Cao Z, Mountjoy KG *et al* (1999) Stimulation of tumors to synthesize tumor necrosis factor-alpha in situ using 5,6-dimethylxanthenone-4-acetic acid: a novel approach to cancer therapy. Cancer Res 59: 633-8.

Jung H-A, Su B-N, Keller WJ *et al* (2006) Antioxidant Xanthones from the Pericarp of Garcinia mangostana (Mangosteen). Journal of Agricultural and Food Chemistry 54: 2077-2082.

Kalialis LV, Drzewiecki KT & Klyver H (2009) Spontaneous regression of metastases from melanoma: review of the literature. Melanoma Res 19: 275-82.

Korn EL, Liu PY, Lee SJ *et al* (2008) Meta-analysis of phase II cooperative group trials in metastatic stage IV melanoma to determine progression-free and overall survival benchmarks for future phase II trials. J Clin Oncol 26: 527-34.

Kostakis IK, Magiatis P, Pouli N *et al* (2002) Design, synthesis, and antiproliferative activity of some new pyrazole-fused amino derivatives of the pyranoxanthenone, pyranothioxanthenone, and pyranoacridone ring systems: a new class of cytotoxic agents. J Med Chem 45: 2599-609.

Kouklakis G, Efremidou EI, Pitiakoudis M *et al* (2013) Development of primary malignant melanoma during treatment with a TNF-alpha antagonist for severe Crohn's disease: a case report and review of the hypothetical association between TNF-alpha blockers and cancer. Drug Des Devel Ther 7: 195-9.

Krasagakis K, Tholke D, Farthmann B *et al* (1998) Elevated plasma levels of transforming growth factor (TGF)-beta1 and TGF-beta2 in patients with disseminated malignant melanoma. Br J Cancer 77: 1492-4.

Kumar IV, Paul BN, Asthana R *et al* (2003) Swertia chirayita mediated modulation of interleukin-1beta, interleukin-6, interleukin-10, interferon-gamma, and tumor necrosis factor-alpha in arthritic mice. Immunopharmacol Immunotoxicol 25: 573-83.

Kundu JK & Surh YJ (2008) Cancer chemopreventive and therapeutic potential of resveratrol: mechanistic perspectives. Cancer Lett 269: 243-61.

Kundu N & Fulton AM (1997) Interleukin-10 inhibits tumor metastasis, downregulates MHC class I, and enhances NK lysis. Cell Immunol 180: 55-61.

Kuo PC & Schroeder RA (1995) The emerging multifaceted roles of nitric oxide. Ann Surg 221: 220-35.

Kurzrock R (2001) Cytokine deregulation in cancer. Biomed Pharmacother 55: 543-7.

Kushnir I & Merimsky O (2013) The evolution in melanoma treatment as a reflection of precision-oriented medicine. Oncol Lett 5: 424-426.

Lakshmi Narendra B, Eshvendar Reddy K, Shantikumar S *et al* (2013) Immune system: a double-edged sword in cancer. Inflamm Res 62: 823-34.

Lala PK & Orucevic A (1998) Role of nitric oxide in tumor progression: lessons from experimental tumors. Cancer Metastasis Rev 17: 91-106.

Lara PN, Jr., Douillard JY, Nakagawa K *et al* (2011) Randomized phase III placebo-controlled trial of carboplatin and paclitaxel with or without the vascular disrupting agent vadimezan (ASA404) in advanced non-small-cell lung cancer. J Clin Oncol 29: 2965-71.

Le Gal FA, Avril MF, Bosq J *et al* (2001) Direct evidence to support the role of antigen-specific CD8(+) T cells in melanoma-associated vitiligo. J Invest Dermatol 117: 1464-70.

Leiro J, Arranz JA, Yanez M *et al* (2004) Expression profiles of genes involved in the mouse nuclear factor-kappa B signal transduction pathway are modulated by mangiferin. Int Immunopharmacol 4: 763-78.

Leiro JM, Alvarez E, Arranz JA *et al* (2003) In vitro effects of mangiferin on superoxide concentrations and expression of the inducible nitric oxide synthase, tumour necrosis factor-alpha and transforming growth factor-beta genes. Biochem Pharmacol 65: 1361-71.

Li Y & Ohizumi Y (2004) Search for constituents with neurotrophic factor-potentiating activity from the medicinal plants of paraguay and Thailand. Yakugaku Zasshi 124: 417-24.

Lin CN, Chung MI, Liou SJ *et al* (1996) Synthesis and anti-inflammatory effects of xanthone derivatives. J Pharm Pharmacol 48: 532-8.

Lin W-W & Karin M (2007) A cytokine-mediated link between innate immunity, inflammation, and cancer. The Journal of Clinical Investigation 117: 1175-1183.

Liu J, Fukunaga-Kalabis M, Li L *et al* (2014) Developmental pathways activated in melanocytes and melanoma. Arch Biochem Biophys.

Liu SH, Lee LT, Hu NY *et al* (2012) Effects of alpha-mangostin on the expression of anti-inflammatory genes in U937 cells. Chin Med 7: 19.

Liu Z, Antalek M, Nguyen L *et al* (2013) The effect of gartanin, a naturally occurring xanthone in mangosteen juice, on the mTOR pathway, autophagy, apoptosis, and the growth of human urinary bladder cancer cell lines. Nutr Cancer 65 Suppl 1: 68-77.

Madan B, Singh I, Kumar A *et al* (2002) Xanthones as inhibitors of microsomal lipid peroxidation and TNF-alpha induced ICAM-1 expression on human umbilical vein endothelial cells (HUVECs). Bioorg Med Chem 10: 3431-6.

Maio M (2012) Melanoma as a model tumour for immuno-oncology. Ann Oncol 23 Suppl 8: viii10-4.

Makare N, Bodhankar S & Rangari V (2001) Immunomodulatory activity of alcoholic extract of Mangifera indica L. in mice. J Ethnopharmacol 78: 133-7.

Makitie T, Summanen P, Tarkkanen A *et al* (2001) Tumor-infiltrating macrophages (CD68(+) cells) and prognosis in malignant uveal melanoma. Invest Ophthalmol Vis Sci 42: 1414-21.

Mantovani A & Sica A (2010) Macrophages, innate immunity and cancer: balance, tolerance, and diversity. Curr Opin Immunol 22: 231-7.

Mantovani A, Sica A, Sozzani S *et al* (2004) The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 25: 677-86.

Mantovani A, Sozzani S, Locati M *et al* (2002) Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 23: 549-55.

Mariette X, Matucci-Cerinic M, Pavelka K *et al* (2011) Malignancies associated with tumour necrosis factor inhibitors in registries and prospective observational studies: a systematic review and meta-analysis. Ann Rheum Dis 70: 1895-904.

Martinez FO, Helming L & Gordon S (2009) Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol 27: 451-83.

Matsumoto K, Akao Y, Kobayashi E *et al* (2003) Induction of apoptosis by xanthones from mangosteen in human leukemia cell lines. Journal of Natural Products 66: 1124-1127.

Matsumoto K, Akao Y, Ohguchi K *et al* (2005) Xanthones induce cell-cycle arrest and apoptosis in human colon cancer DLD-1 cells. Bioorg Med Chem 13: 6064-9.

Matsumoto K, Akao Y, Yi H *et al* (2004) Preferential target is mitochondria in α-mangostin-induced apoptosis in human leukemia HL60 cells. Bioorganic & Medicinal Chemistry 12: 5799-5806.

Matzinger P (2002) The danger model: a renewed sense of self. Science 296: 301-5.

Mazimba O, Nana F, Kuete V *et al* (2013) 11 - Xanthones and Anthranoids from the Medicinal Plants of Africa. In*:* KUETE, V. (ed.) Medicinal Plant Research in Africa. Oxford: Elsevier.

Mccourt C, Dolan O & Gormley G (2014) Malignant Melanoma: A Pictorial Review. Ulster Med J 83: 103-110.

Mckeage MJ, Reck M, Jameson MB *et al* (2009) Phase II study of ASA404 (vadimezan, 5,6-dimethylxanthenone-4-acetic acid/DMXAA) 1800mg/m(2) combined with carboplatin and paclitaxel in previously untreated advanced non-small cell lung cancer. Lung Cancer 65: 192-7.

Meyer C, Sevko A, Ramacher M *et al* (2011) Chronic inflammation promotes myeloid-derived suppressor cell activation blocking antitumor immunity in transgenic mouse melanoma model. Proc Natl Acad Sci U S A 108: 17111-6.

Moncada S, Palmer RM & Higgs EA (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 43: 109-42.

Moongkarndi P, Kosem N, Kaslungka S *et al* (2004) Antiproliferation, antioxidation and induction of apoptosis by Garcinia mangostana (mangosteen) on SKBR3 human breast cancer cell line. Journal of Ethnopharmacology 90: 161-166.

Morrison CD, Parvani JG & Schiemann WP (2013) The relevance of the TGF-beta Paradox to EMT-MET programs. Cancer Lett 341: 30-40.

Munk ME & Emoto M (1995) Functions of T-cell subsets and cytokines in mycobacterial infections. Eur Respir J Suppl 20: 668s-675s.

Murray PJ & Wynn TA (2011) Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol 11: 723-37.

Nakagawa Y, Iinuma M, Naoe T *et al* (2007) Characterized mechanism of α-mangostin-induced cell death: Caspase-independent apoptosis with release of endonuclease-G from mitochondria and increased miR-143 expression in human colorectal cancer DLD-1 cells. Bioorganic and Medicinal Chemistry 15: 5620-5628.

Nakatani K, Atsumi M, Arakawa T *et al* (2002) Inhibitions of histamine release and prostaglandin E2 synthesis by mangosteen, a Thai medicinal plant. Biol Pharm Bull 25: 1137-41.

Parker DC (1993) T cell-dependent B cell activation. Annu Rev Immunol 11: 331-60.

Pedraza-Chaverri J, Cárdenas-Rodríguez N, Orozco-Ibarra M *et al* (2008) Medicinal properties of mangosteen (Garcinia mangostana). Food and Chemical Toxicology 46: 3227-3239.

Pedro M, Cerqueira F, Sousa ME *et al* (2002) Xanthones as inhibitors of growth of human cancer cell lines and their effects on the proliferation of human lymphocytes in vitro. Bioorganic & medicinal chemistry 10: 3725-30.

Pfister JR, Ferraresi RW, Harrison IT *et al* (1972) Xanthone-2-carboxylic acids, a new series of antiallergic substances. J Med Chem 15: 1032-5.

Phan GQ, Attia P, Steinberg SM *et al* (2001) Factors associated with response to high-dose interleukin-2 in patients with metastatic melanoma. J Clin Oncol 19: 3477-82.

Pinto MM, Sousa ME & Nascimento MS (2005) Xanthone derivatives: new insights in biological activities. Curr Med Chem 12: 2517-38.

Porta C, Subhra Kumar B, Larghi P *et al* (2007) Tumor promotion by tumor-associated macrophages. Adv Exp Med Biol 604: 67-86.

Printz C (2001) Spontaneous regression of melanoma may offer insight into cancer immunology. J Natl Cancer Inst 93: 1047-8.

Qian BZ & Pollard JW (2010) Macrophage diversity enhances tumor progression and metastasis. Cell 141: 39-51.

Raimondi G, Turner MS, Thomson AW *et al* (2007) Naturally occurring regulatory T cells: recent insights in health and disease. Crit Rev Immunol 27: 61-95.

Rajendran P, Ekambaram G, Magesh V *et al* (2008) Chemopreventive efficacy of mangiferin against benzo(a)pyrene induced lung carcinogenesis in experimental animals. Environmental Toxicology and Pharmacology 26: 278-282.

Reiman JM, Kmieciak M, Manjili MH *et al* (2007) Tumor immunoediting and immunosculpting pathways to cancer progression. Semin Cancer Biol 17: 275-87.

Romagnani S, Parronchi P, D'elios MM *et al* (1997) An update on human Th1 and Th2 cells. Int Arch Allergy Immunol 113: 153-6.

Roncarolo MG, Bacchetta R, Bordignon C *et al* (2001) Type 1 T regulatory cells. Immunol Rev 182: 68-79.

Roncarolo MG, Gregori S, Battaglia M *et al* (2006) Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. Immunol Rev 212: 28-50.

Sabat R, Grutz G, Warszawska K *et al* (2010) Biology of interleukin-10. Cytokine Growth Factor Rev 21: 331-44.

Santibanez JF, Quintanilla M & Bernabeu C (2011) TGF-beta/TGF-beta receptor system and its role in physiological and pathological conditions. Clin Sci (Lond) 121: 233-51.

Seo EK, Kim NC, Wani MC *et al* (2002) Cytotoxic prenylated xanthones and the unusual compounds anthraquinobenzophenones from Cratoxylum sumatranum. J Nat Prod 65: 299-305.

Shan T, Ma Q, Guo K *et al* (2011) Xanthones from mangosteen extracts as natural chemopreventive agents: potential anticancer drugs. Curr Mol Med 11: 666-77.

Sica A & Mantovani A (2012) Macrophage plasticity and polarization: in vivo veritas. J Clin Invest 122: 787-95.

Siveen KS & Kuttan G (2009) Role of macrophages in tumour progression. Immunol Lett 123: 97-102.

Solinas G, Germano G, Mantovani A *et al* (2009) Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. J Leukoc Biol 86: 1065-73.

Speeckaert R, Van Geel N, Vermaelen KV *et al* (2011) Immune reactions in benign and malignant melanocytic lesions: lessons for immunotherapy. Pigment Cell Melanoma Res 24: 334-44.

Spurrell EL & Lockley M (2014) Adaptive immunity in cancer immunology and therapeutics. Ecancermedicalscience 8: 441.

Suksamrarn S, Komutiban O, Ratananukul P *et al* (2006) Cytotoxic prenylated xanthones from the young fruit of Garcinia mangostana. Chemical and Pharmaceutical Bulletin 54: 301-305.

Sun J, Chu YF, Wu X *et al* (2002) Antioxidant and antiproliferative activities of common fruits. J Agric Food Chem 50: 7449-54.

Tang YP, Li PG, Kondo M *et al* (2009) Effect of a mangosteen dietary supplement on human immune function: a randomized, double-blind, placebo-controlled trial. J Med Food 12: 755-63.

Teixeira M, Cerqueira F, Barbosa CM *et al* (2005) Improvement of the inhibitory effect of xanthones on NO production by encapsulation in PLGA nanocapsules. J Drug Target 13: 129-35.

Tewtrakul S, Wattanapiromsakul C & Mahabusarakam W (2009) Effects of compounds from Garcinia mangostana on inflammatory mediators in RAW264.7 macrophage cells. J Ethnopharmacol 121: 379-82.

Tsutsui S, Yasuda K, Suzuki K *et al* (2005) Macrophage infiltration and its prognostic implications in breast cancer: the relationship with VEGF expression and microvessel density. Oncol Rep 14: 425-31.

U. F (2012) Regulation of nitric oxide synthase expression and activity. In*:* MAYER, B. (ed.) Nitric Oxide. Springer Berlin Heidelberg.

Varney ML, Johansson SL & Singh RK (2005) Tumour-associated macrophage infiltration, neovascularization and aggressiveness in malignant melanoma: role of monocyte chemotactic protein-1 and vascular endothelial growth factor-A. Melanoma Res 15: 417-25.

Vesely MD, Kershaw MH, Schreiber RD *et al* (2011) Natural innate and adaptive immunity to cancer. Annu Rev Immunol 29: 235-71.

Vieira LM & Kijjoa A (2005) Naturally-occurring xanthones: recent developments. Curr Med Chem 12: 2413-46.

Wang JJ, Sanderson BJS & Zhang W (2011) Cytotoxic effect of xanthones from pericarp of the tropical fruit mangosteen (Garcinia mangostana Linn.) on human melanoma cells. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 49: 2385-91.

Watanapokasin R, Jarinthanan F, Jerusalmi A *et al* (2010) Potential of Xanthones from Tropical Fruit Mangosteen as Anti-cancer Agents: Caspase-Dependent Apoptosis Induction In Vitro and in Mice. Applied Biochemistry and Biotechnology 162: 1080-1094.

Weecharangsan W, Opanasopit P, Sukma M *et al* (2006) Antioxidative and neuroprotective activities of extracts from the fruit hull of mangosteen (Garcinia mangostana Linn.). Med Princ Pract 15: 281-7.

Wiemann B & Starnes CO (1994) Coley's toxins, tumor necrosis factor and cancer research: a historical perspective. Pharmacol Ther 64: 529-64.

Wilson CB, Rowell E & Sekimata M (2009) Epigenetic control of T-helper-cell differentiation. Nat Rev Immunol 9: 91-105.

Wink DA, Kasprzak KS, Maragos CM *et al* (1991) DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. Science 254: 1001-3.

Wink DA, Vodovotz Y, Laval J *et al* (1998) The multifaceted roles of nitric oxide in cancer. Carcinogenesis 19: 711-21.

Woo CH, Eom YW, Yoo MH *et al* (2000) Tumor necrosis factor-alpha generates reactive oxygen species via a cytosolic phospholipase A2-linked cascade. J Biol Chem 275: 32357-62.

Yao YB, Peng ZG, Liu ZF *et al* (2010) [Effects of mangiferin on cell cycle status and CDC2/Cyclin B1 expression of HL-60 cells]. Zhong yao cai = Zhongyaocai = Journal of Chinese medicinal materials 33: 81-85.

Yusuf-Makagiansar H, Anderson ME, Yakovleva TV *et al* (2002) Inhibition of LFA-1/ICAM-1 and VLA-4/VCAM-1 as a therapeutic approach to inflammation and autoimmune diseases. Med Res Rev 22: 146-67.

Zeng L, O'connor C, Zhang J *et al* (2010) IL-10 promotes resistance to apoptosis and metastatic potential in lung tumor cell lines. Cytokine 49: 294-302.

Zhang JM & An J (2007) Cytokines, inflammation, and pain. Int Anesthesiol Clin 45: 27-37.

Zhou L, Chong MM & Littman DR (2009) Plasticity of CD4+ T cell lineage differentiation. Immunity 30: 646-55.

Ziche M & Morbidelli L (2000) Nitric oxide and angiogenesis. J Neurooncol 50: 139-48.

Zijlmans HJ, Fleuren GJ, Baelde HJ *et al* (2006) The absence of CCL2 expression in cervical carcinoma is associated with increased survival and loss of heterozygosity at 17q11.2. J Pathol 208: 507-17.

Zou YS, Hou AJ, Zhu GF *et al* (2004) Cytotoxic isoprenylated xanthones from Cudrania tricuspidata. Bioorg Med Chem 12: 1947-53.

II. State of art

Xanthones as potential agents in melanoma treatment

Melanoma mortality rates are a devastating reality and a concern for investigators and physicians. The main reason for the poor outcome is the few treatment options. Standard treatments, chemo and radiotherapy, are inefficient in many cases (Flaherty 2010); alternative therapies as BRAF inhibitors are only useful for patients with the mutation and either for a relative short time (Solit and Rosen 2011); ipilimumab, an immunomodulating antibody that target CTLA4 on activated T cells and inhibit regulatory T cells, exhibit severe toxicity (Hodi *et al.* 2010; Graziani *et al.* 2012).

Clearly, the achievement of an efficient therapeutic agent in melanoma patients is desirable that to act in multiple targets in different biochemical pathways to avoid tumor escape mechanisms (Smalley *et al.* 2006).

Taking into account all biologic activities that have been associated with xanthone derivatives, lead some of them to be interesting candidates for melanoma treatment.

In fact, a great variety of xanthones showed significant antitumor activity against human and murine melanoma cell lines and also *in vivo*. Antitumor activity of xanthones from mangosteen were reported against human melanoma SK-MEL-28 (Wang *et al.* 2011; Wang *et al.* 2012a; Wang *et al.* 2012b; Wang *et al.* 2013) and, most recently, in highly metastatic murine melanoma B16-F10 and in A375 human melanoma (Beninati *et al.* 2014). Similar effects have been reported for other natural xanthones (Azebaze *et al.* 2007) and synthetic xanthones (Valenti *et al.* 1993; Joseph *et al.* 1999; Chen *et al.* 2000; Pedro *et al.* 2002; Sousa *et al.* 2002). In spite of and as far as we know, there are no studies in α-MG effect on melanoma cells growth by SRB assay.

Natural xanthones has been shown remarkable activities in the improvement of human health, particularly in cancer. However, the biochemical pathways restrict the possible structures of xanthones and do not allow taking the full potential of these compounds. In order to overtake this limitation, some investigations try to find the relationship between structure and biological activities. Pedro *et al* established some of these important relationships associated to different xanthone compounds and concluded that their effects depend on the nature of substituents, the position where these are placed in the xanthonic nucleus and, sometimes, depends on the cell type. The knowledge of these interactions is an important step for achieve a xanthonic derivative more specific and efficient for particular diseases and decrease the potential side effects of natural xanthones (view chapter V: paper 1).

Importance of immunomodulation in melanoma treatment

As previously referred, several evidences highlighted the importance of immunomodulation of tumor microenvironment in the control of melanoma.

The reported immunologic effects of xanthones are not restricted to few pathways; these compounds influence either innate or adaptive immune system. Their particular effect on NO and cytokines production by macrophages was already attested. Natural xanthones inhibited NO production and TNF-α secretion by murine macrophages cell lines (Chen *et al.* 2008; Tewtrakul *et al.* 2009; Boonnak *et al.* 2014). In U397 human macrophages-like cell lines, natural compounds decreased the expression of inflammatory genes (as TNF-α and IL-6) and inhibited the Th1 and Th2 differentiation (Bumrungpert *et al.* 2010; Liu *et al.* 2012). Contrariwise, Gutierrez-Orozco *et al*. described an over expression of TNF-α by primary monocyte-derived macrophages treated with alpha-mangostin (Gutierrez-Orozco *et al.* 2013). *In vivo* results confirm the anti-inflammatory activities in several rat models (Shankaranarayan *et al.* 1979; Jang *et al.* 2012). Nevertheless, the experiments in humans, even being scarce, showed inconsistencies and some pro-inflammatory cytokines levels, as IL-1β and complement components, were increased, while other markers of inflammation, as C-reactive protein level, were reduced (Tang *et al.* 2009; Udani *et al.* 2009). NO inhibition in murine macrophages was also described for synthetic xanthones (Teixeira *et al.* 2005).

In spite of the verified interaction of immune system in melanoma treatment and the potential of xanthones as antitumor and immunomodulatory agents, any research was based on the direct effect of xanthone in the immune microenvironment of tumor.

Macrophages play an essential role in malignancies (Chen *et al.* 2011) and they are fundamental agents for activation of adaptive immune system (Heidenreich 1999). According to that, study of macrophage involvement on immune microenvironment of tumor appeared to be an important start, in particular to analyze of the xanthones outcome in malignancies.

Immune system is a well regulated network, T lymphocytes are able to modulate the polarization of macrophages and, in turn, macrophages control the response of T lymphocytes (Hao *et al.* 2012; Sica and Mantovani 2012). According to that reciprocal interaction, lymphocytes may interfere with macrophages response in cancer and the effect of xanthones on these immune cells functions is also important for antitumor studies.

Effect of xanthones from mangosteen in cytokines release by lymphocytes from peripheral blood was described only once. Kasemwattanaroj *et al* showed that alpha-mangostin did not induce the production of pro-inflammatory cytokines (TNF-α and IL-1β)

or cytokines of adaptive immunity (IL-2) in lymphocytes (Kasemwattanaroj *et al.* 2013). A research in healthy humans, using 59 mL per day for 30 days of a xanthone-rich mangosteen supplement, increased the ratio of T helper to cytotoxic cells (Tang *et al.* 2009). However, in tumor murine models exposed to gambogic acid, a natural prenylated xanthone, induced an activation of T lymphocytes and a consequent induction of tumor apoptosis (Gu *et al.* 2008).

Other natural xanthones showed divergent function in lymphocytes proliferation: xanthones from *Calophyllum teysmannii* var. *inuphylloide* inhibit their proliferation (Gonzalez *et al.* 1999), while xanthones from *Gentiana barbata* activated their proliferation and induced the formation of specific cytolytic T-killers *(*Nikolaeva *et al.* 2004*)*. Some synthetic xanthones also showed anti-proliferative potential in lymphocytes isolated from peripheral blood (Pedro *et al.* 2002; Sousa *et al.* 2002).

The controversial data concerning the immunomodulatory activity of xanthones needs to be clarified in order to understand the potential of these compounds in cancer treatment, namely in melanoma due to it immunogenic character.

References

Azebaze AG, Dongmo AB, Meyer M *et al* (2007) Antimalarial and vasorelaxant constituents of the leaves of Allanblackia monticola (Guttiferae). Ann Trop Med Parasitol 101: 23-30.

Beninati S, Oliverio S, Cordella M *et al* (2014) Inhibition of cell proliferation, migration and invasion of B16-F10 melanoma cells by alpha-mangostin. Biochem Biophys Res Commun.

Boonnak N, Chantrapromma S, Tewtrakul S *et al* (2014) Inhibition of nitric oxide production in lipopolysaccharide-activated RAW264.7 macrophages by isolated xanthones from the roots of Cratoxylum formosum ssp. pruniflorum. Arch Pharm Res.

Bumrungpert A, Kalpravidh RW, Chuang CC *et al* (2010) Xanthones from mangosteen inhibit inflammation in human macrophages and in human adipocytes exposed to macrophage-conditioned media. J Nutr 140: 842-7.

Chen LG, Yang LL & Wang CC (2008) Anti-inflammatory activity of mangostins from Garcinia mangostana. Food Chem Toxicol 46: 688-93.

Chen P, Huang Y, Bong R *et al* (2011) Tumor-associated macrophages promote angiogenesis and melanoma growth via adrenomedullin in a paracrine and autocrine manner. Clinical cancer research : an official journal of the American Association for Cancer Research 17: 7230-9.

Chen Y-L, Chen IL, Tzeng C-C *et al* (2000) Synthesis and Cytotoxicity Evaluation of Certain α-Methylidene- γ-butyrolactones Bearing Coumarin, Flavone, Xanthone, Carbazole, and Dibenzofuran Moieties. Helvetica Chimica Acta 83: 989-994.

Flaherty K (2010) Advances in drug development. BRAF validation in melanoma. Clin Adv Hematol Oncol 8: 31-4.

Gonzalez MJ, Nascimento MS, Cidade HM *et al* (1999) Immunomodulatory Activity of Xanthones from Calophyllum teysmannii var. inuphylloide. Planta Med 65: 368-71.

Graziani G, Tentori L & Navarra P (2012) Ipilimumab: A novel immunostimulatory monoclonal antibody for the treatment of cancer. Pharmacological Research 65: 9-22.

Gu H, You Q, Liu W *et al* (2008) Gambogic acid induced tumor cell apoptosis by T lymphocyte activation in H22 transplanted mice. Int Immunopharmacol 8: 1493-502.

Gutierrez-Orozco F, Chitchumroonchokchai C, Lesinski GB *et al* (2013) alpha-Mangostin: anti-inflammatory activity and metabolism by human cells. J Agric Food Chem 61: 3891-900.

Hao N-B, Lü M-H, Fan Y-H *et al* (2012) Macrophages in Tumor Microenvironments and the Progression of Tumors. Clinical and Developmental Immunology 2012: 11.

Heidenreich S (1999) Monocyte CD14: a multifunctional receptor engaged in apoptosis from both sides. J Leukoc Biol 65: 737-43.

Hodi FS, O'day SJ, Mcdermott DF *et al* (2010) Improved Survival with Ipilimumab in Patients with Metastatic Melanoma. New England Journal of Medicine 363: 711-723.

Jang HY, Kwon OK, Oh SR *et al* (2012) Mangosteen xanthones mitigate ovalbumin-induced airway inflammation in a mouse model of asthma. Food Chem Toxicol 50: 4042-50.

Joseph WR, Cao Z, Mountjoy KG *et al* (1999) Stimulation of tumors to synthesize tumor necrosis factor-alpha in situ using 5,6-dimethylxanthenone-4-acetic acid: a novel approach to cancer therapy. Cancer Res 59: 633-8.

Kasemwattanaroj P, Moongkarndi P, Pattanapanyasat K *et al* (2013) Immunomodulatory activities of alpha-mangostin on peripheral blood mononuclear cells. Nat Prod Commun 8: 1257-60.

Liu SH, Lee LT, Hu NY *et al* (2012) Effects of alpha-mangostin on the expression of anti-inflammatory genes in U937 cells. Chin Med 7: 19.

Nikolaeva GG, Sergeev AV, Nikolaev SM *et al* (2004) Isolation and Immunomodulant Activity of Gentiabavaroside from Gentiana barbata. Pharmaceutical Chemistry Journal 38: 25-27.

Pedro M, Cerqueira F, Sousa ME *et al* (2002) Xanthones as inhibitors of growth of human cancer cell lines and their effects on the proliferation of human lymphocytes in vitro. Bioorganic & medicinal chemistry 10: 3725-30.

Shankaranarayan D, Gopalakrishnan C & Kameswaran L (1979) Pharmacological profile of mangostin and its derivatives. Arch Int Pharmacodyn Ther 239: 257-69.

Sica A & Mantovani A (2012) Macrophage plasticity and polarization: in vivo veritas. J Clin Invest 122: 787-95.

Smalley KS, Haass NK, Brafford PA *et al* (2006) Multiple signaling pathways must be targeted to overcome drug resistance in cell lines derived from melanoma metastases. Mol Cancer Ther 5: 1136-44.

Solit DB & Rosen N (2011) Resistance to BRAF Inhibition in Melanomas. New England Journal of Medicine 364: 772-774.

Sousa EP, Silva AMS, Pinto MMM *et al* (2002) Isomeric Kielcorins and Dihydroxyxanthones: Synthesis, Structure Elucidation, and Inhibitory Activities of Growth of Human Cancer Cell Lines and on the Proliferation of Human Lymphocytes In Vitro. Helvetica Chimica Acta 85: 2862-2876.

Tang YP, Li PG, Kondo M *et al* (2009) Effect of a mangosteen dietary supplement on human immune function: a randomized, double-blind, placebo-controlled trial. J Med Food 12: 755-63.

Teixeira M, Cerqueira F, Barbosa CM *et al* (2005) Improvement of the inhibitory effect of xanthones on NO production by encapsulation in PLGA nanocapsules. J Drug Target 13: 129-35.

Tewtrakul S, Wattanapiromsakul C & Mahabusarakam W (2009) Effects of compounds from Garcinia mangostana on inflammatory mediators in RAW264.7 macrophage cells. J Ethnopharmacol 121: 379-82.

Udani JK, Singh BB, Barrett ML *et al* (2009) Evaluation of Mangosteen juice blend on biomarkers of inflammation in obese subjects: a pilot, dose finding study. Nutr J 8: 48.

Valenti P, Da Re P, Rampa A *et al* (1993) Benzo-gamma-pyrone analogues of geiparvarin: synthesis and biological evaluation against B16 melanoma cells. Anticancer Drug Des 8: 349-60.

Wang JJ, Sanderson BJ & Zhang W (2012a) Significant anti-invasive activities of alpha-mangostin from the mangosteen pericarp on two human skin cancer cell lines. Anticancer Res 32: 3805-16.

Wang JJ, Sanderson BJS & Zhang W (2011) Cytotoxic effect of xanthones from pericarp of the tropical fruit mangosteen (Garcinia mangostana Linn.) on human melanoma cells. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 49: 2385-91.

Wang JJ, Shi QH, Zhang W *et al* (2012b) Anti-skin cancer properties of phenolic-rich extract from the pericarp of mangosteen (Garcinia mangostana Linn.). Food and Chemical Toxicology 50: 3004-3013.

Wang JJ, Zhang W & Sanderson BJ (2013) Altered mRNA expression related to the apoptotic effect of three xanthones on human melanoma SK-MEL-28 cell line. Biomed Res Int 2013: 715603.

III. Objectives and outline

The main goal of this project was the evaluation of two xanthone derivatives, alpha-mangostin and 1,2-dihydroxyxanthone, in modulation of macrophages activity as well as the result in cytotoxic effect on melanoma cell line, since macrophages are key cells in tumor immune microenvironment.

Therefore, the following were required:

- Evaluate the antitumor activity of referred compounds in the growth of A375-C5 human melanoma cell line by SRB assay;
- Measure the effects of xanthone-induced macrophage-conditioned medium on the proliferation of the human melanoma cell line A375-C5 by SRB assay;
- Compare the effects of the compounds and the macrophages-conditioned medium on the growth of A375-C5 cell line;
- Evaluate the interference of the compounds with NO production by LPS-stimulated RAW 264.7 murine macrophages, using Griess reagent;
- Access the impact of compounds on the cytokine expression profile by PMA-differentiated THP-1 cells and their polarization into M1 (IL-1 and TNF-α) or M2 (IL-10 and TGF-β1) phenotype, by an ELISA kit;
- Determine the antiproliferative effect of compounds on PHA stimulated human mononuclear cells by MTT assay and their interference with cytokines expression, using an ELISA kit.
- Define THP-1, RAW 264.7 and human mononuclear cells viability after treatment with the compounds by MTT-assay.

IV. Materials and Methods

Chemicals and reagents

Reagents used in cell culture, including RPMI-1640, DMEM medium, and fetal bovine serum (FBS) were purchased from Gibco® Invitrogen Co. (Barcelona, Spain), 2-mercaptoethanol for synthesis was obtained from Merck (Whitehouse Station, NJ, USA), Glutamine cell culture grade and Dimethyl sulfoxide (DMSO) from Applichem (Darmstadt, Germany). N.N-Dimetilformamida (DMF) was acquiring from Spectramol Science Incorporated.

Reagents used in SRB assay as Trichloroacetic acid (TCA) and Acetic acid glacial 99-100% were purchased from Prolabo (Oeiras, Portugal) and CHEM-LAB (Zedelgem, Belgium), respectively.

IL-1β, lL-10, TNF-α and TGF-β1 ELISA Ready-Set-Go Kits were acquired from eBioscience (San Diego, CA, USA).

All other chemicals of analytical grade used in the experiments and unless otherwise indicated were purchased from Sigma-Aldrich® (St. Louis, MO, USA).

Xanthones

Alpha-mangostin (α-MG) was obtained from Sigma-Aldrich® (ref.M3824) and 1,2-dihydroxyxanthone (1,2-DHX) was synthetized in Laboratory of Organic and Pharmaceutic Chemistry (Faculty of Pharmacy, University of Porto) and in CEQUIMED (Centro de Química Medicinal; University of Porto) as previously described (Gales *et al.* 2001)

Stock solution of compounds kept at -20ºC in DMSO. Just prior each assay, stock solutions were diluted in appropriate complete medium to the maximum concentration desired to test and 1:2 dilutions were serially prepared.

Cell lines

A375-C5 human malignant melanoma cell line and RAW 264.7 mouse macrophage cell line are part of CEQUIMED cell culture collection. THP-1 human monocyte cell line was a courtesy of Rui Appelberg.

A375-C5 was routinely maintained in 25 cm^2 flasks containing RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1 µL mL^{-1} Gentamycin at 37ºC in a humidified incubator with 5% CO_2. For the THP-1 cell line the culture medium just described was also supplemented with 0.05 mM 2-mercaptoethanol. RAW 264.7 cell line was maintained in 75 cm^2 flasks containing DMEM medium with the same percentage of FBS and gentamycin, and at the same conditions.

Cell lines were sub-cultured every 2 or 3 days by trypsinization (A375-C5), replacement of the medium (THP-1) or scraping (RAW 264.7) and used for each experiment when the cells were in exponential growth.

Human mononuclear cells were isolated from peripheral blood of healthy volunteers by Histopaque-1077 density centrifugation. All volunteers assign an informed consent. Mononuclear cells were ressuspended in RPMI medium supplemented with 10% FBS and 1 µL mL^{-1} gentamycin.

Cell growth assay

The effect of α-MG on the growth of A375-C5 cell line was determined by sulphorrodamine B (SRB) method adopted by the National Cancer Institute (NCI, USA) (Monks *et al.* 1991) and as already described by our group (Pedro *et al.* 2002; Gupta *et al.* 2008).

A375-C5 cell line was plated into 96-well flat-bottom tissue culture plates at a concentration of 7,5 x 10^4 cells mL^{-1} in complete culture medium and incubated for 24 h in a 5% CO_2 humidified incubator (HERA cell 150, Heraeus) at 37ºC. Once adherent, cells of one of the plates were immediately fixed for a no-growth control (designed Tzero plate) to determine the basal protein amount. Simultaneously, cell of test plates were treated with serial dilutions of xanthones and incubated for another 48h.

Cells were then fixed with 50 µL of 50% TCA solution and incubated for 1h at 4ºC. Cells were washed five times with deionized water and allowed to dry. Fixed cells were exposed to 50 µL SRB dye (0,4% w/v in 1% acetic acid) for 30 minutes at room temperature. The unbound dye was removed by wash five times with 1% (v/v) acetic acid manually. Once completely dried, 100 µL of 10 mM Tris base solution was added and left for 30 minutes at room temperature to solubilize SRB. The absorbance was measured in an ELISA reader (Stat Fax 3200, Awareness Technology). Doxorubicin (1:10 dilutions) was used as positive control.

Growth inhibition of 50% (GI_{50}) was calculated by comparing, after 48 h, the absorbance of the xanthone-treated cells with the absorbance of Tzero plate. Lower absorbance after 48 h of treatment indicate occurrence of cell death instead of growth arrest (Monks *et al.* 1991).

Antitumor effect of conditioned macrophages culture medium

THP-1 human monocyte cell line is a simple, suitable and reliable model to study monocytes and macrophages functions or responses and the possible effects from external stimuli as new drugs. Differentiation of this cell line in mature THP-1 monocyte-derived macrophages can be achieve using phorbol-12-myristate-13-acetate (PMA), the most efficient differentiation agent in this case (Chanput et al. 2012; Chanput et al. 2014).

THP-1 cell line was plated at 1 X 10^5 cells mL^{-1} in 96-wells flat-bottom plates and differentiated into THP-1 macrophages by adding 50 µL of 10 ng mL^{-1} PMA for 72h in a humidified incubator at 37ºC and 5% CO_2 (He *et al.* 2012). Once differentiated, cells were washed twice with complete medium and they were incubated for another 24h in culture medium to obtain the resting stages of macrophages (Chanput *et al.* 2012; Chanput *et al.* 2014). Then, they were stimulated by 100 µL of a solution of 1 µg mL^{-1} LPS, simultaneously treated with the xanthone in test and incubated for 24h at 37ºC in a 5% CO_2 humidified incubator (He *et al.* 2012). Plates were centrifuged to deposit the compounds and half of the volume of each well was added to A375-C5 adherent cells, previously plated as described in section “cell growth assay”. After a 48h incubation, SRB assay was performed, absorbance was measured and cell-growth inhibition determinate as just described (Pedro *et al.* 2002; Gupta *et al.* 2008).

NO production assay

The effect of the different compounds in nitric oxide (NO) production by RAW 264.7 was determined by the Griess reaction as previous described by our group (Teixeira *et al.* 2005). RAW 264.7 (1 X 10^6 cells mL^{-1}) were platted on 96-well flat-bottom plates and allowed to adhere for 2h. Supernatants were removed and replaced by 100 µL of fresh medium containing 1.5 µg mL^{-1} LPS in order to induce iNOS synthesis by macrophages. The cell were also treated with 100 µL of each xanthone concentration (1:2 dilution) and incubated for 24h at 37ºC in a 5% CO_2 humidified incubator. Then, 100 µL of supernatants were transferred to a new 96-well flat-bottom plates, 100 µL of Griess reagent (some quantities of a solution of 1% w/v sulphanilamide in 5% v/v phosphoric acid and a solution of 0.1% w/v naphtylethylenediamide in deionized water) was added and it was incubated for 10 min at room temperature (Green *et al.* 1982). Nitrite production was quantified by spectrophotometry at 550nm in an ELISA reader. *N*-nitro-L-arginine methyl ester (L-NAME) (inhibitor of iNOS activity) and Dexamethasone (inhibitor of iNOS expression) were used as positive controls. Inhibition of 50% NO production was measured by comparing percentage of NO in treated cells and in non-treated cells.

Compounds were also added 6h or 14h after LPS stimulation and significant differences detected in NO production between the different moments when xanthones were added, works to infer if compounds act in iNOS expression and/or activity.

NO scavenging assay

To discard NO scavenging effect by xanthones, nitrite was chemically generated, as previously described (Teixeira *et al.* 2005). 50 µL of sodium nitoprusside (5 mM) in PBS was added to xanthone dilution and incubated for 150 min at 25ºC. Griess reagent was added and nitrite accumulation was quantified. Sodium nitoprusside in PBS (100% production) and in ethanol/PBS (9:1; 0% production) were used as controls.

Scavenging activity, determined in terms of percentage of nitrite formation, was present when the percentage of nitrite formed in the presence of the sample was less than 70% of the sodium nitroprusside control.

Human mononuclear cells MTT-proliferation assay

Human mononuclear cells (50 µl of a concentration of 2-3 X 10^6 cell mL^{-1}) were plated in 96-well flat-bottom plates, treated with 50 µL of serial dilutions of α-MG and stimulated with 50 µL of PHA (Phytohaemagglutinin; used as a mitogen to trigger T-lymphocyte cell division) (10 µg mL^{-1}) and incubated for 96h at 37ºC in a 5% CO2 humidified incubator. After incubation, 25 µL of 1 mg mL^{-1} MTT solution (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) was added to cells. After 4h, the formazan product was solubilized overnight with a sodium lauryl sulfate/N,N-dimethylformamide (SDS/DMF) solution and absorbance measured at 550 nm (Pedro *et al.* 2002; Cerqueira *et al.* 2003; Teixeira *et al.* 2005). Cyclosporin A was used as positive control. Lymphocyte toxicity, determined in terms of the percentage of viable cells, was present when the viability of the treated cells, compared with that of the non-treated control cells, was less than 70%.

Cytokine quantification

Culture supernatants of THP-1 differentiated, stimulated and treated cells, as previously described in section “Antitumor effect of conditioned macrophages culture medium”, were storage at - 20ºC until cytokine analyses.

Levels of interleukin-1β (IL-1β), interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α) and transforming growth Factor-β1 (TGF-β1) in culture supernatants were quantified by

ELISA, using ELISA Ready-SET-Go kit (affymetrix, ebioscience) according to manufacturers' instructions.

The same cytokines were also evaluated in supernatants of human mononuclear cells treated with xanthones for 48h (Lou *et al.* 2013; Wang *et al.* 2013), incubation with PHA stimulus and three concentrations of α-MG.

Interference of both compounds or α-MG in cytokines expression by THP-1 and lymphocytes, respectively, was accessed comparing treated with non-treated cells.

MTT-viability assay

Viability assays were performed as described elsewhere by our group (Cerqueira *et al.* 2003; Teixeira *et al.* 2005).

For lymphocytes, MTT-viability assay was performed after 24h of cells treatment with xanthones. For all the other cells, viability assay was executed at the end of the experiments to determine the effect of the compound on the cell line.

Briefly, to cells supernatants, 25 µL of MTT solution was added followed by 4 h incubation at 37ºC in a 5% CO_2 humidified incubator. After incubation, 50 µL of SDS/DMF solution was added and absorbance measured at 550 nm (Pedro *et al.* 2002; Cerqueira *et al.* 2003; Teixeira *et al.* 2005).

Cell toxicity was considered when the viability of the exposed cells was less than 70%.

Statistical analysis

Except otherwise stated, results are the mean ± SEM of at least three independent experiments, performed in duplicate.

Statistical analysis was performed with SPSS for Windows (version 20.0). Statistical significance between two mean was calculated by Mann-Whitney Test and it is considered for p values less than 0.05.

Ethics

Ethics approved was obtained by the Ethic Comity of University Fernando Pessoa.

References

Cerqueira F, Cordeiro-Da-Silva A, Araujo N *et al* (2003) Inhibition of lymphocyte proliferation by prenylated flavones: artelastin as a potent inhibitor. Life Sci 73: 2321-34.

Chanput W, Mes JJ & Wichers HJ (2014) THP-1 cell line: An in vitro cell model for immune modulation approach. Int Immunopharmacol.

Chanput W, Reitsma M, Kleinjans L *et al* (2012) beta-Glucans are involved in immune-modulation of THP-1 macrophages. Mol Nutr Food Res 56: 822-33.

Gales L, Sousa MED, Pinto MMM *et al* (2001) Naturally occurring 1,2,8-trimethoxyxanthone and biphenyl ether intermediates leading to 1,2-dimethoxyxanthone. Acta Crystallographica Section C 57: 1319-1323.

Green LC, Wagner DA, Glogowski J *et al* (1982) Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem 126: 131-8.

Gupta S, Rodrigues LM, Esteves AP *et al* (2008) Synthesis of N-aryl-5-amino-4-cyanopyrazole derivatives as potent xanthine oxidase inhibitors. Eur J Med Chem 43: 771-80.

He X, Shu J, Xu L *et al* (2012) Inhibitory effect of Astragalus polysaccharides on lipopolysaccharide-induced TNF-a and IL-1beta production in THP-1 cells. Molecules 17: 3155-64.

Lou X, Hou Y & Liang D (2013) Effects of hepatitis B virus X protein on human T cell cytokines. Can J Microbiol 59: 620-6.

Monks A, Scudiero D, Skehan P *et al* (1991) Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. J Natl Cancer Inst 83: 757-66.

Pedro M, Cerqueira F, Sousa ME *et al* (2002) Xanthones as inhibitors of growth of human cancer cell lines and their effects on the proliferation of human lymphocytes in vitro. Bioorganic & medicinal chemistry 10: 3725-30.

Teixeira M, Cerqueira F, Barbosa CM *et al* (2005) Improvement of the inhibitory effect of xanthones on NO production by encapsulation in PLGA nanocapsules. J Drug Target 13: 129-35.

Wang H, Daniel V, Sadeghi M *et al* (2013) Differences in the induction of induced human CD4(+) CD25(+) FoxP3(+) T-regulatory cells and CD3(+) CD8(+) CD28(-) T-suppressor cells subset phenotypes in vitro: comparison of phorbol 12-myristate 13-acetate/ionomycin and phytohemagglutinin stimulation. Transplant Proc 45: 1822-31.

V. Natural Xanthones: alpha-mangostin

Papers 1 and 2

Paper 1 (submitted to Fitoterapia in 29-09-2014): Mangosteen extract: "angel or demon"? The role of xanthones of mangosteen in potential adverse effects

Mangosteen extract: "angel or demon"? The role of xanthones of mangosteen in potential adverse effects

Viviana Silva [a, b]; Rui Medeiros [a, c, d, e]; Fátima Cerqueira [b, d, f, *] & Madalena Pinto [f, g, *]

[a] Instituto de Ciências Biomédicas Abel Salazar (ICBAS), University of Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal;
[b] FP-ENAS/CEBIMED, Faculty of Health Sciences, University of Fernando Pessoa, Ruas Carlos da Maia, 296, 4200-150 Porto, Portugal;
[c] Molecular Oncology GRP and Virology LB, Portuguese Institute of Oncology-Porto, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal;
[d] Faculty of Health Sciences of Fernando Pessoa University, Ruas Carlos da Maia, 296, 4200-150 Porto, Portugal;
[e] LPCC, Portuguese League Against Cancer, Regional Centre of the North, Estrada Interior da Circunvalação, 6657, 4200- 177 Porto, Portugal.
[f] Centro de Química Medicinal da Universidade do Porto (CEQUIMED-UP) and Laboratory of organic and Pharmaceutical Chemistry, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal;
[g] Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto, Porto, Portugal;

***** Corresponding authors:

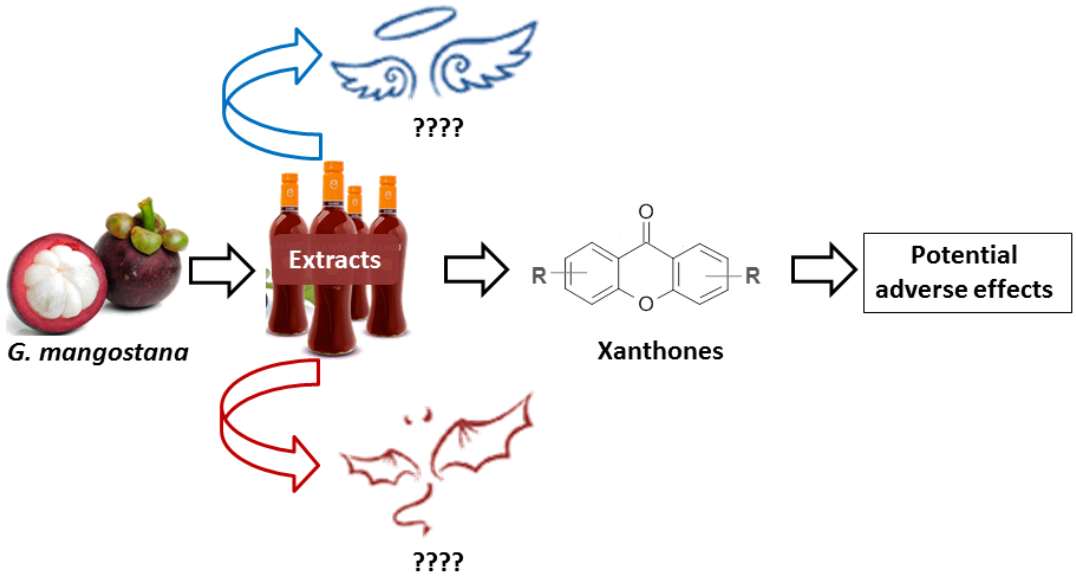
F. Cerqueira
Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Ruas Carlos da Maia, 296, 4200-150 Porto, Portugal
E-mail: fatimaf@ufp.edu.pt
Phone: +351 225074630
Fax: +351 225074637

M. Pinto
Laboratório de Química Orgânica e Farmacêutica, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal
E-mail: madalena@ff.up.pt
Phone: +351 220428692
Fax: +351 226093390

Abstract

Extracts and derived products of the fruit of the mangosteen tree (*Garcinia mangostana* Linn) are among the top-selling dietary supplements due to a great variety of proclaimed pharmacological/biological activities, namely anti-inflammatory, antioxidant, anticancer, among others. This fruit (mangosteen) comprises a huge variety of compounds, being xanthones the main secondary metabolites and the major responsible for the mangosteen declared beneficial characteristics. The knowledge about effects of xanthones from mangosteen at cellular and molecular level has been increasing and, despite all health improvement indications, have many controversial outcomes. The present review focus on potential adverse effects that can be anticipated by continuous and/or excessive use of these products. In fact, xanthones from mangosteen can interfere with important physiologic functions as immunity, cell cycle progression, detoxification and response to serotonin, and may change the metabolism of several drugs by altering the activity of drug-metabolizing enzymes. Finally, the discussion reinforces the importance of ample studies to assure the safety and efficacy of mangosteen derived products for human consumption.

Graphical abstract



Key words: Adverse effects; Dietary supplements; *Garcinia mangostana* Linn; Mangosteen; Xanthones;

Abbreviations
AP-1 – activator protein 1
CAT – catalase
cdc2 – cell division cycle protein 2 homolog
CDK4 – cyclin-dependent kinases 4
CHEK2 – checkpoint kinase 2
COX – cyclooxygenase
CPK – creatine phosphokinase
CYP – cytochrome P450;
eNOS – endothelial nitric oxide synthases
GOT – glutamate oxaloacetate transaminase
GPT – glutamate pyruvate transaminase
GPx – glutathione peroxidase
GSH – glutathione
GST – glutathione-S-transferase
IL – interleukins;
iNOS – inducible nitric oxide synthases
LDH – lactate dehydrogenase
LPS – lipopolysaccharides
MAPKs – mitogen-activated protein kinases
MCP-1 – monocyte chemoattractant protein-1
MDM2 – murine doble minute 2
NF-κB – nuclear factor kappa B
NO – nitric oxide
$p21^{cip1}$ – cyclin-dependent kinase inhibitor 1
p27 – cyclin-dependent kinase inhibitor
p53 – tumor protein p53
SOD – superoxide dismutase
STAT-1 – signal transducer and activator of transcription 1
TLR-2 – Toll-like receptor-2
TNF-α – tumour necrosis factor α

1. Introduction

The use of plants, parts of plants and isolated secondary metabolites for the prevention and treatment of various illnesses has been time long-established. *Garcinia mangostana* Linn is a tropical tree that belongs to the family Clusiaceae, genus *Garcinia* has been cultivated for centuries in Southeast Asia and different parts, mostly fruit hull, bark and roots, have been used in folk medicine to treat a great variety of conditions like infections, wounds, inflammations, dysenteries, among others (Obolskiy *et al.* 2009; Gutierrez-Orozco and Failla 2013) and is not an exception in this area. Its fruit, mangosteen, comprises a huge variety of compounds including xanthones, flavonoids, triterpenoids, benzophenones, terpenes, anthocyanins, tannins, phenols as well as several vitamins (Kosem *et al.* 2007; Chin and Kinghorn 2008). In spite of that, xanthones are the most representative secondary metabolites of *G*. *mangostana* with associated medicinal properties (Chin and Kinghorn 2008; Obolskiy *et al.* 2009).

The interest in mangosteen, especially in xanthone derivatives may be illustrated by a few important reviews (Pinto *et al.* 2005; Obolskiy *et al.* 2009; Shan *et al.* 2011; Gutierrez-Orozco and Failla 2013). A great variety of biological/pharmacological properties, as antioxidant (Jung *et al.* 2006), antitumor (Matsumoto *et al.* 2003; Moongkarndi *et al.* 2004; Suksamrarn *et al.* 2006; Nakagawa *et al.* 2007; Akao *et al.* 2008; Watanapokasin *et al.* 2010; Chao *et al.* 2011; Krajarng *et al.* 2011; Wang *et al.* 2011; Yoo *et al.* 2011; Johnson *et al.* 2012; Chitchumroonchokchai *et al.* 2013; Leão *et al.* 2013; Wang *et al.* 2013), immunomodulatory (Tang *et al.* 2009), cardioprotective (Shankaranarayan *et al.* 1979; Devi Sampath and Vijayaraghavan 2007), neuroprotective (Weecharangsan *et al.* 2006), antiallergic (Nakatani *et al.* 2002a), antimicrobial (Chen *et al.* 1996; Suksamrarn *et al.* 2003; Sakagami *et al.* 2005; Kaomongkolgit *et al.* 2009) analgesic (Cui *et al.* 2010) and anti-inflammatory (Nakatani *et al.* 2002b; Chen *et al.* 2008) have been described.

The remarkable proclaimed advantages of this plant called the attention of some industries that soon started to commercialize a large number of food supplements, beverages and others food products containing mangosteen and occasionally others juices, advertising for their multiples benefits for human health (Garrity *et al.* 2004).

Although herbal medicinal products have been generally considered as relatively low risk by the public, potential damage for health can occurs. Very often attention is not given to the fact that toxicity also can be associated with natural products, by themselves or by contamination, adulteration, plant misidentification, and interactions with other herbal products or drugs (Marcason 2006; Jordan *et al.* 2010; Ibrahim *et al.*) leading to an increased toxicity or loss of therapeutic efficacy (Colalto 2010).

During last decades increased reports on adverse effects regulatory/monitoring agencies in many countries have given alerts for these kind of issues (Shi and Klotz 2012; Sahoo and Manchikanti 2013), leading to increased attention by the scientific community involved in the areas of use of medicinal plants (Foti and Wahlstrom 2008).

The present review emerged from research concerning cellular and molecular mechanisms modulated by xanthones from mangosteen and focuses on potential adverse effects of those xanthones on human physiological pathways. We considered this approach is critical because the intake of such products has been increasing even without any clinical trials that attest their safety or efficacy to human use.

2. Xanthones from mangosteen

Before discussing the interference of xanthones from mangosteen in the human body that could lead to damages, it is important to understand some chemical characteristics of these metabolites.

Xanthones are a class of heterocyclic compounds characterized by a dibenzo-γ-pyrone nucleus (Pedro *et al.* 2002; Pinto *et al.* 2005; Pinto and Castanheiro 2009; Mazimba *et al.* 2013). They are composed by a unique chemical scaffold, the tricyclic aromatic system (C_6–C_3–C_6) named xanthene-9-one (**Figure 6**) (Pinto *et al.* 2005; Teixeira *et al.* 2005; Obolskiy *et al.* 2009; Pinto and Castanheiro 2009).

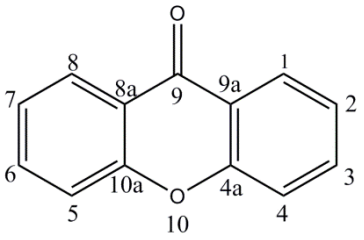


Figure 6 Xanthone basic skeleton

Natural xanthones can be categorized, depending on the nature of the substituents, into simple oxygenated, glycosylated, prenylated and their derivatives (xanthone dimers, xanthonolignoids and miscellaneous) (Pinto *et al.* 2005; Pinto and Castanheiro 2009).

The main xanthones isolated from the different parts of *G. mangostana* are α- and γ-mangostin, but besides these more than 60 compounds were identified, being the most abundant and frequently studied compounds represented in **Figure 7** (Chin and Kinghorn 2008; Obolskiy *et al.* 2009; Pinto and Castanheiro 2009; Shan *et al.* 2011; Leão *et al.* 2013).

Xanthones of the mangosteen extract are subjected to metabolic clearance mediated essentially by multiple cytochrome P450 (CYP) isoforms. CYP1A2 metabolizes α-mangostin, 9-hydroxycalabaxanthones and 8-deoxygartanin; β-Mangostin is mainly metabolized by CYP2C9 and while other isoforms, including CYP2B6, CYP2C19, CYP3A4 and CYP2D6 get a minor contribution; gartanin is as well metabolized by CYP1A2, CYP2B6 and CYP2D6; 3-isomangostin appeared to be only metabolized by CYP3A4 (Foti *et al.* 2009). However, it is important to emphasize that the metabolism can vary if it occurs in normal or malignant cells (Gutierrez-Orozco and Failla 2013).

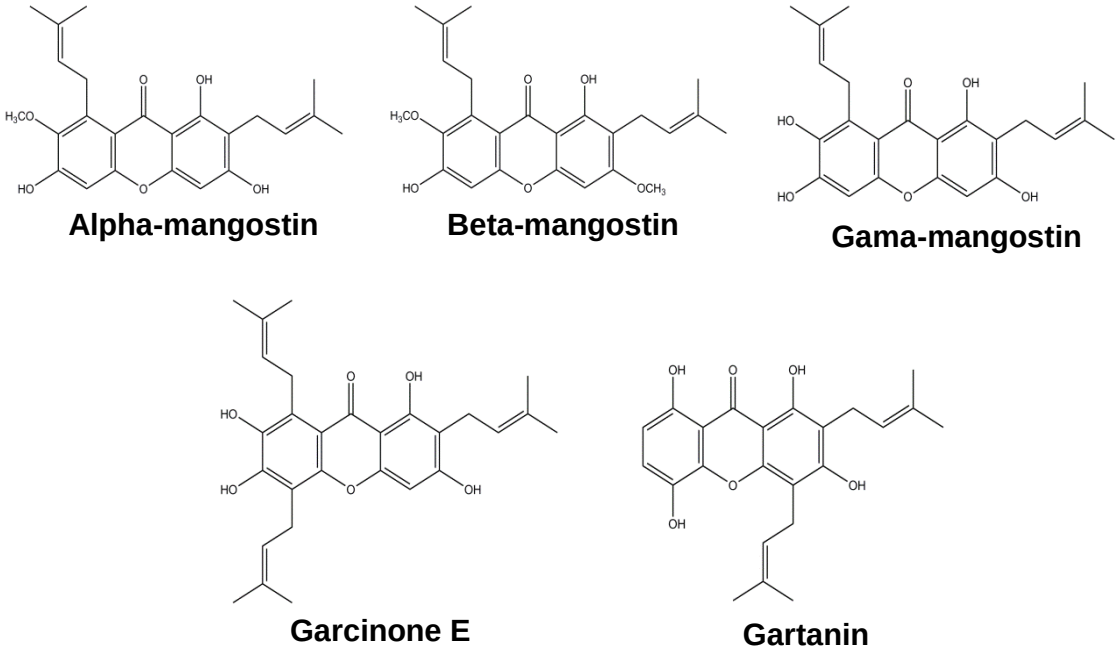


Figure 7 Structure of the main xanthones from mangosteen

3. Potential adverse effects associated with mangosteen

Taking in consideration that herbal medicines are used as whole plants or plant extracts, consisting in combinations of many substances, often with undefined composition, the toxicological assessment of these products is not an easy task, even though the evaluation of the toxicity of the isolated metabolites can be considered routine. If this happens it will be of great interest to correlate the toxicity of these compounds with the extract. Although this is not the usual state, there are already examples of this type of correlation (Jordan *et al.* 2010).

With *Garcinia mangostana* this kind of study has not been done and one of the purposes of this review in the next chapters is to point to the influence of the potential toxicity of xanthones from mangosteen, in order to give clues for a future correlation with the adverse effects associated with mangosteen extracts.

3.1. Immunomodulation

Nowadays, many induced alterations on the immune system has been described and associated with potential benefits for many illnesses as diabetes, cardiac pathologies, cancer and autoimmune diseases (Frangogiannis *et al.* 2002; Sampath and Vijayaragavan 2008; Bumrungpert *et al.* 2009a; Dougan and Dranoff 2009; Hanahan and Weinberg 2011; Singh *et al.* 2012). However, the immune system comprises large and complex pathways with antagonist and/or synergic functions that

change according to several conditions as age, gender, stress, environment, among others. Consequently, the immune response is hard to predict and interpret (Cohen *et al.* 1991; Lesourd 2002; Beery 2003; Srinivasan *et al.* 2005). In cancer, for example, the innate and adaptive immunity have a dual effect, either preventing tumour progression or stimulating their growth, invasiveness and metastatic activity (Dranoff 2004; Gutkin and Shurin 2014).

The immune function is essential for organism homeostasis, responding to exogenous and endogenous risk signals (Matzinger 2002). Therefore, the uncontrolled and unmeasured use of antiphlogistic agents, as mangosteen products, may cause severe adverse effects in consumers.

Exploring recent reports of immunomodulation mediated by these products, we noticed that many of these possibly trigger body deregulation.

The inhibition of pro-inflammatory genes triggered by lipopolysaccharides (LPS) was recurrently described and related with xanthones exposure in different cell lines. Many authors reported a decrease in gene expression of tumour necrosis factor α (TNF-α), several interleukins (IL-1β, IL-6, IL-8), monocyte chemoattractant protein-1 (MCP-1) and Toll-like receptor-2 (TLR-2) in primary cultures of human adipocytes, human U937 macrophages-like cells and murine RAW 264.7 macrophage-like cells treated with α- and/or γ-mangostin (Sampath and Vijayaragavan 2008; Bumrungpert *et al.* 2009a; Bumrungpert *et al.* 2009b; Tewtrakul *et al.* 2009; Bumrungpert *et al.* 2010; Liu *et al.* 2012; Gutierrez-Orozco *et al.* 2013). But this effect is not considered to be exclusive of macrophages and adipocytes, Gutierrez-Orozco *et al*. detected similar inhibition, mediated by α-mangostin, in human monocyte cell line (THP-1) and different cancer cell lines (HepG2, Caco-2 HTB-37, and HT-29) (Gutierrez-Orozco *et al.* 2013). The described alterations on pro-inflammatory genes seems to be associated with the interference in some immune pathways, those involving conventional mitogen-activated protein kinases (MAPKs), activator protein 1 (AP-1), signal transducer and activator of transcription 1 (STAT-1) and nuclear factor kappa B (NF-κB) (Sampath and Vijayaragavan 2008; Bumrungpert *et al.* 2009a; Tewtrakul *et al.* 2009; Liu *et al.* 2012). These protein cascades are also involved in stress response and participate in crosstalk with other signal pathways (Ghosh *et al.* 1998; Ramana *et al.* 2000; Chang and Karin 2001; Li and Verma 2002; Bonizzi and Karin 2004; Wada and Penninger 2004) which means that the effect of xanthones from mangosteen, namely α- and/or γ-mangostin, will probably not be confined to immune system modulation.

Additional restriction on immunity cells action seems to occur at basophils and mast cells level. Xanthonic compounds (α-, β- and/or γ-mangostin) and extracts of mangosteen reduce degranulation of these cells preventing histamine release possibly by interfering with downstream signals (Nakatani *et al.* 2002a; Itoh *et al.* 2008). Moreover, the histaminergic receptors are blocked by α and γ-mangostin (Chairungsrilerd *et al.* 1996). As consequence of this blockage, these compounds deregulate the interaction histamine-receptors, a binding implicated in some pathological functions including cancer and intestinal ischemia promotion (Kusche *et al.* 1980; Raithel *et al.* 1998), along with several vital functions, as neurotransmission, immunomodulation, haematopoiesis, wound healing and day-night rhythm (Maintz and Novak 2007). According to that, a careful medical follow-up is needed to prevent possible complications.

Anti-inflammatory activity of mangosteen extracts and α- and/or γ-mangostin have been still linked with enzymatic alterations, namely through inhibition of the mRNA expression and/or activity of cyclooxygenases 1 and 2 (COX-1 and COX-2) and/ or inducible nitric oxide synthases (iNOS) (Nakatani *et al.* 2002b; Nakatani *et al.* 2004; Chen *et al.* 2008; Tewtrakul *et al.* 2009). COX-1 and COX-2 are involved in physiologic processes as well as pathophysiologic events. COX-1 is traditionally associated with homeostasis functions, including gastric cytoprotection and haemostasis. COX-2 is connected to inflammation and tumourigenesis. In spite of this, complementary functions between the two enzymes and variations on their actions depending of the specific target organs were reported (Masferrer *et al.* 1994; Dubois *et al.* 1998; Marnett 2009; Rouzer and Marnett 2009). Inducible nitric oxide synthases (iNOS) is responsive for nitric oxide (NO) production after stimulation by immunological stimuli, being an intermediary of inflammatory response, however the excess or insufficiency of nitric oxide production can cause equally cell damage (Gross and Wolin 1995). In cancer, NO seems to have dual effects: multiple reports has associated NO with cancer progression while others has defended the therapeutic benefits of NO donors in different cell lines and organisms (Wink *et al.* 1991; Lala and Orucevic 1998; Wink *et al.* 1998; Ziche and Morbidelli 2000; Lala and Chakraborty 2001; Choi *et al.* 2002; Mocellin *et al.* 2007; Coulter *et al.* 2008; Yasuda 2008).

Considering the importance of all these enzymes in biochemical and physiological events the role of xanthones contained in mangosteen extracts should be carefully evaluated.

3.2. Cell Cycle arrest

Cell cycle is a sequence of highly regulated events essential for all living beings. In multi-cell organisms are necessary several rounds of cell division to create a new individual and during their lifetime it is a crucial mechanism for maintaining appropriate cellular and tissue condition once cell death and damages are constant (Alberts 2008).

Mechanisms of action of some xanthones from mangosteen are frequently associated with cell cycle arrest. Since the cell cycle deregulation is a hallmark of cancer and one of the earliest processes of oncogenesis (Syljuasen *et al.* 1999; Hanahan and Weinberg 2011), several authors purposed and proved this effect of xanthones in different tumor (Matsumoto *et al.* 2005; Wang *et al.* 2011; Johnson *et al.* 2012; Kurose *et al.* 2012; Leão *et al.* 2013).

Matsumoto *et al.* related that xanthones induced cell-cycle arrest in a human colon cancer cell line. Mangostins α and β up-regulated p27 and down-regulated cyclin A, B1, D1 and E1 and cdc2, phospho-cdc2 (tyrosine 15) expression and consequently act at G1-phase, blocking the division progression. However, while some authors affirm that γ-mangostin acts at S-phase in human colon cancer (Matsumoto *et al.* 2005), others indicate a G1-phase arrest in a human melanoma cell line (Wang *et al.* 2011).

Johnson *et al.* reported that a prostate cancer cell line treated with α-mangostin inhibited cyclins/cyclin-dependent kinases 4 (CDK4) binding, an vital protein complex to cell cycle progression from G1 to S-phase (Johnson *et al.* 2012). According to that, Kurose *et al.* described an up-regulation of $p21^{cip1}$ and CHEK2 (regulator factors of G1-phase) in breast cancer model after treatment with α-mangostin (Kurose *et al.* 2012).

The inhibition of p53-MDM2 interaction due to a potential binding between α-mangostin and MDM2 was also detected by some of our group (Leão *et al.* 2013). Free p53 stops cell division at both G1 and G2 checkpoints (Agarwal *et al.* 1995). In accordance with our finds, further authors have reported enhance of p53 levels after colon and blander cancer treatment with α-mangostin and gartanin, respectively (Aisha *et al.* 2012; Liu *et al.* 2013).

In summary, some xanthones from mangosteen are potent inhibitors of cell cycle progression by blocking the division at different levels in different cell models, as illustrated in **Figure 8**.

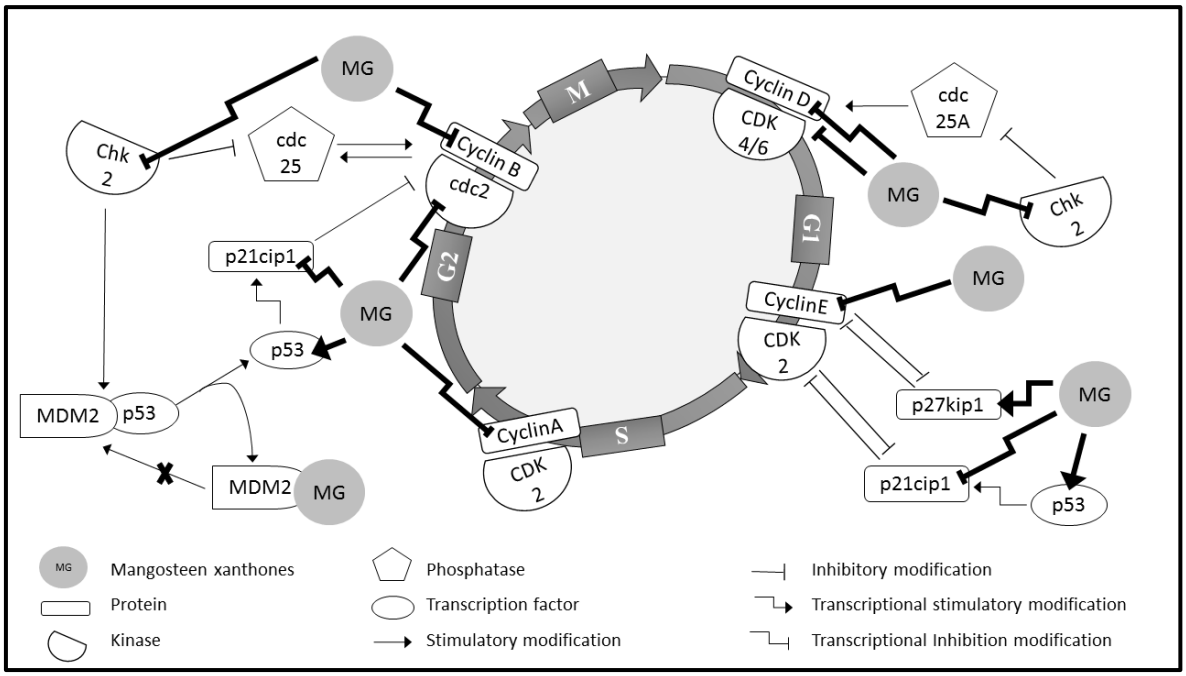


Figure 8 Effect of xanthones of mangosteen at different stages of cell cycle regulation. Legend: cdc indicate cell division cycle protein; CDK, cyclin-dependent kinases; Chk or CHEK2, checkpoint kinase; G1, gap phase; G2, gap phase 2; M, mitosis phase; MDM2, murine doble minute 2; MG, mangosteen xanthones; p21cip1, cyclin-dependent kinase inhibitor 1; p27kip1, cyclin-dependent kinase inhibitor; p53, tumor protein p53; S, DNA synthesis phase.

Besides the importance of that effect in cancer treatment, normal cells have to be replaced to keep the tissue homeostasis, specially, tissues with constitutive division. Labile tissues as hematopoietic cells in the bone marrow and the majority of epithelial tissue (e.g., skin, oral cavity, vagina and cervix, salivary glands, pancreas, biliary and gastrointestinal tracts, uterus, fallopian tubes and urinary tract) must have a functional cell-cycle to replace the lost cells in injuries and aging process (Kumar *et al.* 2012).

Hence that, xanthones of mangosteen referred above (mangostins α, β, γ and gartanin) as cell cycle arrest agents may interfere with normal cells replacement.

3.3. Antioxidant effect

Oxidative stress is a natural process implicated in cell damage or death and it has been associated with several human pathologies, like cardiac and neurodegenerative diseases and cancer (Valko *et al.* 2007). The prevention of such stress through antioxidant products has been extensively study, however the potential benefits or risks associated are still ambiguous (Stanner *et al.* 2004; Bjelakovic *et al.* 2013). In oncologic patients the advantages of antioxidant use are unclear. Some studies admitted survival gains or no interference with chemo or radiotherapy, while others

suggest a reduction of the therapeutic effect of chemo or radiotherapy in patients simultaneous using antioxidant products (D'Andrea 2005; Moss 2007; Lawenda *et al.* 2008; Block *et al.* 2009).

As referred previously, many xanthones from mangosteen have antioxidant activity (Moongkarndi *et al.* 2004; Jung *et al.* 2006; Weecharangsan *et al.* 2006; Devi Sampath and Vijayaraghavan 2007; Kosem *et al.* 2007; Chin *et al.* 2008). In fact, Devi Sampath and Vijayaraghavan 2007 described an attenuation of isoproterenol oxidative effect in rats treated with α-mangostin compared with no-treated rats. They demonstrated an augment of antioxidant enzymes glutathione-S-transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), as well as a reduction of glutathione (GSH), lipid peroxides and serum enzymes, including lactate dehydrogenase (LDH), creatine phosphokinase (CPK), glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) (Devi Sampath and Vijayaraghavan 2007).

In generally, it is known that some drugs, including cancer drug therapies, can be affected by xanthones from mangosteen antioxidant potential. These xanthonic derivatives have been connected with alterations on metabolizing enzymes responsible for the metabolism of several drugs (Foti *et al.* 2009). The changes that can occur in effect of drugs when co-administrated with these compounds will be discussed in the following section.

Considering the referred controversial use of antioxidants, further studies are necessary to confirm the safety of the use of that herbal folk medicine.

3.4. Modifications in the metabolism of therapeutic agents

On living organisms, exogenous compounds are subjected to biochemical modifications catalysed by enzymes responsible for detoxification. Alterations in activity of these drug-metabolizing enzymes may jeopardize the treatment response, namely in efficacy, toxicity or both (Lu 1998; Evans and Relling 2004). Xanthonic components of mangosteen were already associated with modulation of enzymes involved in phase I and phase II of the metabolism (Devi Sampath and Vijayaraghavan 2007; Balunas *et al.* 2008; Foti *et al.* 2009).

The CYP family are phase I enzymes capable to metabolize the majority of drugs (Kalra *et al.* 2009) including natural xanthones, as already was referred. In spite of that, mangosteen has been associated with inhibition of enzymatic activity of some CYP members. Foti *et al.* showed that aqueous mangosteen extract extensible

reduced the activity of CYP2C8 and CYP2C9 in human liver microsomes; further enzymes were also inhibited but to a less extent. These authors proposed that such effect is associated with the presence of α-mangostin, β-mangostin, gartanin, 3-isomangostin and 8-desoxygartanin in the extract (Foti *et al.* 2009). Balunas *et al*. also described a potent inhibition of CYP19 (aromatase) mediated by methanol and chloroform-soluble extracts of *G. mangostana* in a human placental microsomes and by some xanthones (specially γ-mangostin) in a human breast cancer cell line (Balunas *et al.* 2008).

CYP2C8 and 2C9 metabolize important drugs (as indicated in **Table 1**) including the therapeutic agents ibuprofen (Davies 1998; Garcia-Martin *et al.* 2004), paclitaxel, phenytoin (Burns 1999; Foti *et al.* 2009) and warfarin (Burns 1999; Rettie and Tai 2006; Foti *et al.* 2009), as well as amitriptyline (Ghahramani *et al.* 1997; Olesen and Linnet 1997), cyclophosphamide (Chang *et al.* 1993; Chang *et al.* 1997), diazepam (Shou *et al.* 2000) and losartan (Zhou *et al.* 2009; Mannheimer and Eliasson 2010). Therefore, it is predictable that some of these drugs may become less or even ineffective when co-administered with mangosteen products and their toxicity can also be increased.

Similarly, the aromatase inhibition mediated by mangosteen might increase the adverse effects of drugs like methadone (Nekhayeva *et al.* 2005) and hormones androstanolone and nortestosterone (Silberzahn *et al.* 1988; Douglas *et al.* 2005) or reduce the treatment response to others such as methadone derivate levacetylmethadol (Deshmukh *et al.* 2004) and the testosterone (Silberzahn *et al.* 1988; Rendic 2002; Morale *et al.* 2008) (**Table 1**). Additionally to the metabolic activity, CYP19 are also responsible for the bioconversion of androgens into estrogens (Simpson *et al.* 1994). Consequently, the potential of aromatase inhibitors, like mangosteen (α, γ-mangostin and garcinones D/E), in treatment of many hormone-responsive tumors as breast cancer cannot be ignored (Azria *et al.* 2005; Brueggemeier 2006; Kendall and Dowsett 2006).

Beyond the CYP family, the antioxidant effect of α-mangostin by stimulating several enzymes including the glutathione S-transferases (GSTs) (Devi Sampath and Vijayaraghavan 2007) must be considered. GSTs are phase II enzymes that catalyse the conjugation of many endogenous substances and xenobiotics with glutathione (GSH) (Uetrecht and Trager 2007; Oakley 2011; Bousova and Skalova 2012). GSH-conjugation results, normally, in drug activity inhibition due to increase in solubility of compounds and consequently the facility of excretion (Barnouin *et al.* 1998; Peklak-

Scott *et al.* 2005). However in some cases, drug conjugation promotes drug activation (Morgan *et al.* 1998; Findlay *et al.* 2004; Tew 2005). The anticancer drugs, PABA/NO and TLK286 are examples of compounds activated after conjugation (Morgan *et al.* 1998; Findlay *et al.* 2004; Tew 2005) (**Table 1**). Consequently, the co-administration with α-mangostin could promote accumulation of the active metabolites and increasing the side effects.

Table 1. Potential effects of xanthones derivatives on drugs metabolism.

Effect on some drug-metabolizing enzymes	Drugs metabolized by the enzymes #	Therapeutic activity affected	References #
Decrease CYP2C8 and CYP2C9 activity	Amprenavir	Antiretroviral (HIV-1 inhibitor)	(Fung *et al.* 2000)
	Bupropion	Antidepressant tetracyclic	(Preissner *et al.* 2010; Knox *et al.* 2011)
	Capsaicin	Analgesic (natural)	(Reilly *et al.* 2003)
	Cisapride	Gastrointestinal prokinetic agent	(Desta *et al.* 2000; Preissner *et al.* 2010)
	Clozapine *	Antipsychotic	(Fang *et al.* 1998; Rostami-Hodjegan *et al.* 2004)
	Cyamemazine	Antipsychotic and anxiolytic	(Arbus *et al.* 2007)
	Dapsone *	Antibacterial	(Winter *et al.* 2000)
	Dextromethorphan	Cough suppressant	(von Moltke *et al.* 1998; Preissner *et al.* 2010)
	Diclofenac *	Analgesic, antipyretic, anti-inflammatory	(Mancy *et al.* 1999)
	Diltiazem *	Cardiovascular	(Sutton *et al.* 1997)
	Estradiol	Sex hormones (estrogen)	(Yamazaki *et al.* 1998; Cheng *et al.* 2001)
	Fluvastatin *	Lipid regulating	(Scripture and Pieper 2001)
	Ibuprofen *	Non-Steroidal Anti-Inflammatory	(Davies 1998; Garcia-Martin *et al.* 2004)
	Irbesartan *	Cardiovascular	(Hallberg *et al.* 2002; Preissner *et al.* 2010)
	Ketamine	Anesthetic and analgesic	(Hijazi and Boulieu 2002; Preissner *et al.* 2010)
	Ketobemidone	Analgesic	(Kristensen *et al.* 1996; Yasar *et al.* 2005)
	Lansoprazole *	Gastro-intestinal	(Pearce *et al.* 1996)
	Methadone	Analgesic, antipyretic, anti-inflammatory	(Rendic 2002; Zhou *et al.* 2009)
	Omeprazole *	Gastro-intestinal	(Foti *et al.* 2009; Preissner *et al.* 2010)
	Paclitaxel	Antineoplastic	(Burns 1999; Foti *et al.* 2009)
	Perphenazine	Antipsychotic	(Olesen and Linnet 2000; Rendic 2002; Preissner *et al.* 2010)
	Phenytoin *	Antiepileptic	(Burns 1999; Foti *et al.* 2009)
	Phenprocoumon	Cardiovascular	(Ufer *et al.* 2004)
	Piroxicam *	Analgesic, antipyretic, Non-steroidal anti-inflammatory	(Zhao *et al.* 1992; Perini *et al.* 2005; Preissner *et al.* 2010)
	Progesterone *	Sex hormone (Progestagen)	(Yamazaki and Shimada 1997; Preissner *et al.* 2010)
	Propofol *	General anesthetic	(Guitton *et al.* 1998)
	Quinidine *	Cardiovascular	(Nielsen *et al.* 1999; Preissner *et al.* 2010)
	Rosiglitazone *	Antidiabetic	(Baldwin *et al.* 1999; Malinowski and Bolesta 2000)

	Selegiline, Deprenyl *	Antidepressant	(Rendic 2002; Salonen *et al.* 2003)
	Seratrodast *	Cardiovascular	(Kumar *et al.* 1997)
	Temazepam	Anxiolytic, sedative, hypnotic	(Ono *et al.* 1996; Yang *et al.* 1998; Rendic 2002)
	Terbinafine	Antifungal	(Vickers *et al.* 1999; Rendic 2002)
	Thalidomide	Antineoplasic	(Ando *et al.* 2002)
	Tolbutamide *	Antidiabetic	(Srivastava *et al.* 1991; Rendic 2002)
	Torasemide, Torsemide	Cardiovascular (Diuretic)	(Rendic 2002; Kerdpin *et al.* 2004; Vormfelde *et al.* 2004)
	Trimethadione, Troxidone	Antiepileptic	(Kurata *et al.* 1998; Rendic 2002; Tanaka *et al.* 2003)
	Verapamil *	Cardiovascular	(Tracy *et al.* 1999; Anthony and Berg 2002)
	Warfarin*	Cardiovascular (Anticoagulant)	(Burns 1999; Rettie and Tai 2006; Foti *et al.* 2009)
	Zafirlukast *	Bronchodilators and Anti-asthma	(Dekhuijzen and Koopmans 2002; Preissner *et al.* 2010)
	Zopiclone	Anxiolytic, sedative, hypnotic	(Becquemont *et al.* 1999; Rendic 2002)
	Acetylsalicylic acid	Anticoagulants and Antithrombotics	(Preissner *et al.* 2010; Knox *et al.* 2011)
	Amitriptyline*	Antidepressant	(Ghahramani *et al.* 1997; Olesen and Linnet 1997)
	Cyclophosphamide *	Antineoplastic	(Chang *et al.* 1993; Chang *et al.* 1997)
	Diazepam *	Anxiolytic, sedative, hypnotic	(Shou *et al.* 2000)
	Ifosfamide *	Antineoplastic	(Chang *et al.* 1993)
	Lidocain(e)	Local anesthetic	(Narang *et al.* 1978; Rendic 2002)
	Losartan *	Anti-Hypertensives	(Zhou *et al.* 2009; Mannheimer and Eliasson 2010)
	Pioglitazone *	Antidiabetic	(Jaakkola *et al.* 2006; Preissner *et al.* 2010)
	Sulfadiazine *	Antibacterial	(Cribb *et al.* 1995; Rendic 2002)
	Testosterone	Sex hormone (Androgen and anabolic)	(Yamazaki and Shimada 1997; Rendic 2002)
	Troglitazone *	Antidiabetic	(Yamazaki *et al.* 1999b; He *et al.* 2004)
	Zidovudine, azidothymidine	Antiviral	(Eagling *et al.* 1994; Rendic 2002)
	Brompheniramine	Antihistaminic	(Preissner *et al.* 2010; Knox *et al.* 2011)
	Caffeine	Xanthine (CNS stimulant)	(Preissner *et al.* 2010; Knox *et al.* 2011)
	Carbinoxamine	Antihistamine and anticholinergic	(Preissner *et al.* 2010; Knox *et al.* 2011)
	Leflunomide *	Antirheumatic	(Rozman 2002)
	Mefenamic acid *	Analgesic, antipyretic, anti-inflammatory	(Preissner *et al.* 2010; Knox *et al.* 2011)
	Mephenytoin	Antiepileptic	(Relling *et al.* 1990; Goldstein *et al.* 1994)
	Meloxicam *	Analgesic, antipyretic, anti-inflammatory	(Turck *et al.* 1996; Chesne *et al.* 1998; Preissner *et al.* 2010)
	Mirtazapine	Antidepressant	(Stormer *et al.* 2000; Rendic 2002)
	Nicotine	Supplementary drugs and other substances	(Yamazaki *et al.* 1999a; Rendic 2002)
	Paracetamol, acetaminophen	Analgesic, antipyretic, anti-inflammatory	(Raucy *et al.* 1989; Rendic 2002)
	Phenazone, Antipyrine	Analgesic, antipyretic, non-steroidal anti-	(Engel *et al.* 1996; Rendic 2002)

		inflammatory	
	Rifampicin *	Antibacterial	(Preissner *et al.* 2010; Knox *et al.* 2011)
	Rofecoxib	Non-steroidal anti-inflammatory	(Preissner *et al.* 2010; Knox *et al.* 2011)
	Sulfinpyrazon(e) *	Antigout	(Rendic 2002)
	Theophylline	Xanthine (Bronchodilator)	(Preissner *et al.* 2010; Knox *et al.* 2011)
	Trimethoprim *	Antiepileptic	(Rendic 2002)
Decrease CYP19 (aromatase) activity	Betamethason(e) *	Corticosteroid	(Paakki *et al.* 2000)
	Letrozole *	Antineoplastic	(Azria *et al.* 2005)
	Androstanolone	Sex hormones (Androgen)	(Douglas *et al.* 2005)
	Methadone	Analgesic, antipyretic, anti-inflammatory	(Nekhayeva *et al.* 2005)
	Nandrolone, nortestosterone	Sex hormone (Progestagen)	(Silberzahn *et al.* 1988)
	Levacetylmethadol (methadone derivate)	Analgesic, antipyretic, anti-inflammatory	(Deshmukh *et al.* 2004)
	Testosterone	Sex hormone (Androgen and anabolic)	(Silberzahn *et al.* 1988; Rendic 2002; Morale *et al.* 2008)
Increase GST activity	Adriamycin or Doxorubicin	Antineoplastic	(Hayes and Pulford 1995; Lien *et al.* 2002; Hamilton *et al.* 2003)
	Busulfan	Antineoplastic	(Vassord *et al.* 2008; Elhasid *et al.* 2010)
	Carboplatin	Antineoplastic	(Marsh *et al.* 2009)
	Carmustin or BCNU	Antineoplastic	(Hayes and Pulford 1995; Lien *et al.* 2002; Hamilton *et al.* 2003)
	Chlorambucil	Antineoplastic	(Hayes and Pulford 1995; Lien *et al.* 2002; Hamilton *et al.* 2003; Parker *et al.* 2008)
	Cisplatin	Antineoplastic	(Hayes and Pulford 1995; Peters *et al.* 2000; Lien *et al.* 2002; Hamilton *et al.* 2003)
	Cyclophosphamide	Antineoplastic	(Hayes and Pulford 1995; Lien *et al.* 2002; Hamilton *et al.* 2003)
	Ecteinascidin-743; ET-743 or Trabectedin	Antineoplastic	(Brandon *et al.* 2006)
	Ethacrynic acid	Diuretic	(Hayes and Pulford 1995; Lien *et al.* 2002; Hamilton *et al.* 2003)
	Etoposide	Antineoplastic	(Mans *et al.* 1992)
	Glutathione	Supplementary drug	(Hayes *et al.* 2005)
	Melphalan	Antineoplastic	(Hayes and Pulford 1995; Lien *et al.* 2002; Hamilton *et al.* 2003)
	Mitozantrone	Antineoplastic	(Hayes and Pulford 1995; Lien *et al.* 2002; Hamilton *et al.* 2003)
	Oxaliplatin	Antineoplastic	(Marsh *et al.* 2009)
	Thiotepa	Antineoplastic	(Hayes and Pulford 1995; Lien *et al.* 2002; Hamilton *et al.* 2003)
	Azathioprine	Antineoplastic	(Gianluigi Zaza *et al.* 2010)
	TLK286	Antineoplastic	(Morgan *et al.* 1998; Tew 2005)
	PABA/NO	Antineoplastic	(Findlay *et al.* 2004)

Information concerning Cytochrome P450s and Glutathione S-transferases metabolism was obtained from SuperCYP (Preissner *et al.* 2010), DrugBank database (Knox *et al.* 2011) and additional papers; all drugs

indicated on the table are approved by FDA; in drugs metabolized by CYP2C8 and 2C9, only those that are metabolized by the two enzymes was listed because the effect of xanthones might be more significant.
LEGEND: CYP: Cytochrome P450; GST: Glutathione S-transferases; * drugs that, besides substrates, are also inhibitors and/or inducers of enzyme activity; # references related to drugs metabolized by such enzyme.

3.5. Blocking serotonin receptors

Serotonin is a ubiquitous molecule that plays multiple physiological roles including cardiovascular, gastrointestinal and endocrine functions, as well as development, sensory perception, behaviours such as aggression, appetite, sex, sleep, mood, cognition, and memory (Aghajanian and Sanders-Bush 2002). The inhibition of 5-HT_{2A} serotonin receptors mediated by γ-mangostin (Chairungsrilerd *et al.* 1996) was described in animal models; this effect may disrupt several essential functions to human survival.

Serotonergic receptor blocking agents are current drugs used in nervous system disorders (Chairungsrilerd *et al.* 1996) making mangosteen supplements good candidates for intervention at this level. Their use by healthy people however needs to be monitored once this neurotransmitter is intervener in a variety of essential biological processes.

3.6. Other effects

Many others unfavourable proprieties have been linked with the use of mangosteen-based products.

Wong, *et al* described a case of severe lactic acidosis in a woman who took mangosteen juice. The authors postulated that this effect was mediated by α-mangostin causing mitochondrial injuries (Wong and Klemmer 2008). The loss of mitochondrial potential has been described in general by many reviews concerning *G. mangostana* (Pinto *et al.* 2005; Pedraza-Chaverri *et al.* 2008; Obolskiy *et al.* 2009; Shan *et al.* 2011; Chitchumroonchokchai *et al.* 2013; Gutierrez-Orozco and Failla 2013); even if only a case of lactic acidosis was reported until now, associated with an acute respiratory illness, it is necessary to be watchful to understand the possible conditions that improve that risk of its use (Wong and Klemmer 2008).

Several mangosteen products available in market advertise for their antidiabetic potential based on scientific reports. In fact, many authors reported a decrease of insulin-resistance augment of sensibility to insulin in animal or cell models after treatment with xanthones, namely mangiferin and α and γ-mangosteen (Ichiki *et al.* 1998; Miura *et al.* 2001a; Miura *et al.* 2001b; Bumrungpert *et al.* 2009a; Bumrungpert *et al.* 2010). Must be taking in account that mangosteen, as other fruits, contains high

quantities of sugar and since there are no studies in antidiabetic effect in humans, the advantages or either their safety in diabetics cannot be guaranteed. However, mangosteen is processing before commercialized and alterations caused by this treatment may induced modifications in xanthones composition. Moreover, those products contain additional substances, beyond xanthones (Wong and Klemmer 2008).

Finally, FDA (Food and Drugs Administration) alert for the presence of tadalafil in two specific commercialized products. This is a substance that may interact with nitrates found in some prescription drugs (medication frequently used by diabetics and patients who have high blood pressure, high cholesterol or heart diseases) and may lower blood pressure to dangerous levels (FDA 2012). The referred example leads to speculate about a possible addition of non-declared substances in natural products once that an approval is not necessary to their commercialization (FDA 2014).

4. Conclusions

Herbal-based medicines triggered a huge curiosity in past years about their advantages leading to an exponential increasing in marketing.

The potential of *Garcinia mangostana* as a botanic dietary supplement has been encouraged the investigation at several levels and, in this review, we reinforce the fact that mangosteen fruit can show prospective benefits in cancer, diabetes, cardiac, psychiatric, autoimmune and neurodegenerative pathologies, as well as in some physiologic conditions like oxidative stress and inflammation.

In spite of those promising proprieties, dietary supplements based on natural products, like *G. mangostana*, are not subjected to rigorous safety testing in humans and their control is quite limited since is not necessary a FDA's approbation.

Additionally, both safety and efficacy of mangosteen cannot be assured only by scientific studies made in cell lines or animal models as has been done so far. Furthermore, it is necessary taking in account several parameters to make an accurate clinical trial because some biochemical events may alter the mangosteen effects and the availability of their active metabolites. The most important of these parameters are the part of the fruit that would be used, the extraction method, the type and amount of active compounds existent in each extract and the presence of other components that can be add to the mangosteen products, as different juices (blueberry, cranberry, raspberry, grape, cherry, strawberry, pear, apple, etc), green tea (*Camellia sinensis*), *Aloe vera*, multivitamins or essential minerals.

Another point that has received minimal attention is the characterization of the absorption, metabolism and elimination of these compounds in human organism. This characterization is critical for establishing a possible toxicity rate of chronic ingestion of mangosteen and may allow the determination of possible interactions with therapeutic agents. It is also important to verify if the doses of some drugs can be reduced by using mangosteen concomitantly. The evaluation of these parameters may minimize the adverse response to therapy which is extremely important especially for treatments with drugs that cause serious side effects (example of chemotherapeutic drugs as paclitaxel, letrozole or azathioprine).

In summary, this review highlights the importance and the value of extensive animal studies, long-term epidemiologic studies and more controlled clinical trials in human volunteers (Shan *et al.* 2011; Gutierrez-Orozco and Failla 2013) for mangosteen supplements. Certainly, many other food supplements needed similar studies; however mangosteen is one of the top-selling, is easily available, consumed without medical control and can modulate several biochemical pathways. Correct hazard evaluation, along with analysis of related risk factors of its use, are essential to the recognition and avoidance of potential adverse effects. Mangosteen seems to have an enormous potential in improvement human's health, however that only can be truthfully accomplish when all of its effects will be completely understand, what requires a concerted work in several areas like, chemistry, pharmacology, biology and clinic in general.

Acknowledgements

This work was funded by FCT – Fundação para a Ciência e a Tecnologia under the project CEQUIMED-PEST-OE/SAU/UI4040/2014 and Fundação Fernando Pessoa.

5. References

Agarwal ML, Agarwal A, Taylor WR *et al* (1995) p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. Proc Natl Acad Sci U S A 92: 8493-7.

Aghajanian GK & Sanders-Bush E (2002) Neuropsychopharmacology: The Fifth Generation of Progress : an Official Publication of the American College of Neuropsychopharmacology. New York: Lippincott Williams & Wilkins.

Aisha AFA, Abu-Salah KM, Ismail Z *et al* (2012) In vitro and in vivo anti-colon cancer effects of Garcinia mangostana xanthones extract. BMC complementary and alternative medicine 12: 104-104.

Akao Y, Nakagawa Y, Iinuma M *et al* (2008) Anti-cancer effects of xanthones from pericarps of mangosteen. Int J Mol Sci 9: 355-70.

Alberts B (2008) Molecular Biology of the Cell: Fifth edition, Garland Science.

Ando Y, Fuse E & Figg WD (2002) Thalidomide metabolism by the CYP2C subfamily. Clin Cancer Res 8: 1964-73.

Anthony M & Berg MJ (2002) Biologic and molecular mechanisms for sex differences in pharmacokinetics, pharmacodynamics, and pharmacogenetics: Part I. J Womens Health Gend Based Med 11: 601-15.

Arbus C, Benyamina A, Llorca PM *et al* (2007) Characterization of human cytochrome P450 enzymes involved in the metabolism of cyamemazine. Eur J Pharm Sci 32: 357-66.

Azria D, Larbouret C, Cunat S *et al* (2005) Letrozole sensitizes breast cancer cells to ionizing radiation. Breast Cancer Res 7: R156-63.

Baldwin SJ, Clarke SE & Chenery RJ (1999) Characterization of the cytochrome P450 enzymes involved in the in vitro metabolism of rosiglitazone. Br J Clin Pharmacol 48: 424-32.

Balunas MJ, Su B, Brueggemeier RW *et al* (2008) Xanthones from the botanical dietary supplement mangosteen (Garcinia mangostana) with aromatase inhibitory activity. J Nat Prod 71: 1161-6.

Barnouin K, Leier I, Jedlitschky G *et al* (1998) Multidrug resistance protein-mediated transport of chlorambucil and melphalan conjugated to glutathione. Br J Cancer 77: 201-9.

Becquemont L, Mouajjah S, Escaffre O *et al* (1999) Cytochrome P-450 3A4 and 2C8 are involved in zopiclone metabolism. Drug Metab Dispos 27: 1068-73.

Beery TA (2003) Sex differences in infection and sepsis. Crit Care Nurs Clin North Am 15: 55-62.

Bjelakovic G, Nikolova D & Gluud C (2013) Meta-regression analyses, meta-analyses, and trial sequential analyses of the effects of supplementation with beta-carotene, vitamin A, and vitamin E singly or in different combinations on all-cause mortality: do we have evidence for lack of harm? PLoS One 8: e74558.

Block K, Koch A, Mead M *et al* (2009) Re: Should supplemental antioxidant administration be avoided during chemotherapy and radiation therapy? J Natl Cancer Inst 101: 124-5; author reply 125-6.

Bonizzi G & Karin M (2004) The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol 25: 280-8.

Bousova I & Skalova L (2012) Inhibition and induction of glutathione S-transferases by flavonoids: possible pharmacological and toxicological consequences. Drug Metab Rev 44: 267-86.

Brandon EF, Sparidans RW, Guijt KJ *et al* (2006) In vitro characterization of the human biotransformation and CYP reaction phenotype of ET-743 (Yondelis, Trabectidin), a novel marine anti-cancer drug. Invest New Drugs 24: 3-14.

Brueggemeier RW (2006) Update on the use of aromatase inhibitors in breast cancer. Expert Opin Pharmacother 7: 1919-30.

Bumrungpert A, Kalpravidh R, Chitchumroonchokchai C *et al* (2009a) Xanthones from mangosteen prevent lipopolysaccharide-mediated inflammation and insulin resistance in primary cultures of human adipocytes. The Journal of Nutrition: 1185-1191.

Bumrungpert A, Kalpravidh RW, Chuang CC *et al* (2010) Xanthones from mangosteen inhibit inflammation in human macrophages and in human adipocytes exposed to macrophage-conditioned media. The Journal of nutrition 140: 842-7.

Bumrungpert A, Kalpravidh RW, Suksamrarn S *et al* (2009b) Bioaccessibility, biotransformation, and transport of alpha-mangostin from Garcinia mangostana (Mangosteen) using simulated digestion and Caco-2 human intestinal cells. Mol Nutr Food Res 53 Suppl 1: S54-61.

Burns M (1999) Management of narrow therapeutic index drugs. J Thromb Thrombolysis 7: 137-43.

Chairungsrilerd N, Furukawa K, Ohta T *et al* (1996) Histaminergic and serotonergic receptor blocking substances from the medicinal plant Garcinia mangostana. Planta Med 62: 471-2.

Chang L & Karin M (2001) Mammalian MAP kinase signalling cascades. Nature 410: 37-40.

Chang TK, Weber GF, Crespi CL *et al* (1993) Differential activation of cyclophosphamide and ifosphamide by cytochromes P-450 2B and 3A in human liver microsomes. Cancer Res 53: 5629-37.

Chang TK, Yu L, Goldstein JA *et al* (1997) Identification of the polymorphically expressed CYP2C19 and the wild-type CYP2C9-ILE359 allele as low-Km catalysts of cyclophosphamide and ifosfamide activation. Pharmacogenetics 7: 211-21.

Chao AC, Hsu YL, Liu CK *et al* (2011) alpha-Mangostin, a dietary xanthone, induces autophagic cell death by activating the AMP-activated protein kinase pathway in glioblastoma cells. J Agric Food Chem 59: 2086-96.

Chen LG, Yang LL & Wang CC (2008) Anti-inflammatory activity of mangostins from Garcinia mangostana. Food Chem Toxicol 46: 688-93.

Chen SX, Wan M & Loh BN (1996) Active constituents against HIV-1 protease from Garcinia mangostana. Planta Med 62: 381-2.

Cheng ZN, Shu Y, Liu ZQ *et al* (2001) Role of cytochrome P450 in estradiol metabolism in vitro. Acta Pharmacol Sin 22: 148-54.

Chesne C, Guyomard C, Guillouzo A *et al* (1998) Metabolism of Meloxicam in human liver involves cytochromes P4502C9 and 3A4. Xenobiotica 28: 1-13.

Chin YW, Jung HA, Chai H *et al* (2008) Xanthones with quinone reductase-inducing activity from the fruits of Garcinia mangostana (Mangosteen). Phytochemistry 69: 754-8.

Chin YW & Kinghorn AD (2008) Structural Characterization, Biological Effects, and Synthetic Studies on Xanthones from Mangosteen (Garcinia mangostana), a Popular Botanical Dietary Supplement. Mini Rev Org Chem 5: 355-364.

Chitchumroonchokchai C, Thomas-Ahner JM, Li J *et al* (2013) Anti-tumorigenicity of dietary alpha-mangostin in an HT-29 colon cell xenograft model and the tissue distribution of xanthones and their phase II metabolites. Mol Nutr Food Res 57: 203-11.

Choi BM, Pae HO, Jang SI *et al* (2002) Nitric oxide as a pro-apoptotic as well as anti-apoptotic modulator. J Biochem Mol Biol 35: 116-26.

Cohen S, Tyrrell DA & Smith AP (1991) Psychological stress and susceptibility to the common cold. N Engl J Med 325: 606-12.

Colalto C (2010) Herbal interactions on absorption of drugs: Mechanisms of action and clinical risk assessment. Pharmacol Res 62: 207-27.

Coulter JA, Mccarthy HO, Xiang J *et al* (2008) Nitric oxide--a novel therapeutic for cancer. Nitric Oxide 19: 192-8.

Cribb AE, Spielberg SP & Griffin GP (1995) N4-hydroxylation of sulfamethoxazole by cytochrome P450 of the cytochrome P4502C subfamily and reduction of sulfamethoxazole hydroxylamine in human and rat hepatic microsomes. Drug Metab Dispos 23: 406-14.

Cui J, Hu W, Cai Z *et al* (2010) New medicinal properties of mangostins: analgesic activity and pharmacological characterization of active ingredients from the fruit hull of Garcinia mangostana L. Pharmacol Biochem Behav 95: 166-72.

D'andrea GM (2005) Use of antioxidants during chemotherapy and radiotherapy should be avoided. CA Cancer J Clin 55: 319-21.

Davies NM (1998) Clinical pharmacokinetics of ibuprofen. The first 30 years. Clin Pharmacokinet 34: 101-54.

Dekhuijzen PN & Koopmans PP (2002) Pharmacokinetic profile of zafirlukast. Clin Pharmacokinet 41: 105-14.

Deshmukh SV, Nanovskaya TN, Hankins GD *et al* (2004) N-demethylation of levo-alpha-acetylmethadol by human placental aromatase. Biochem Pharmacol 67: 885-92.

Desta Z, Soukhova N, Mahal SK *et al* (2000) Interaction of cisapride with the human cytochrome P450 system: metabolism and inhibition studies. Drug Metab Dispos 28: 789-800.

Devi Sampath P & Vijayaraghavan K (2007) Cardioprotective effect of alpha-mangostin, a xanthone derivative from mangosteen on tissue defense system against isoproterenol-induced myocardial infarction in rats. J Biochem Mol Toxicol 21: 336-9.

Dougan M & Dranoff G (2009) Immune therapy for cancer. Annu Rev Immunol 27: 83-117.

Douglas JA, Zuhlke KA, Beebe-Dimmer J *et al* (2005) Identifying susceptibility genes for prostate cancer--a family-based association study of polymorphisms in CYP17, CYP19, CYP11A1, and LH-beta. Cancer Epidemiol Biomarkers Prev 14: 2035-9.

Dranoff G (2004) Cytokines in cancer pathogenesis and cancer therapy. Nat Rev Cancer 4: 11-22.

Dubois RN, Abramson SB, Crofford L *et al* (1998) Cyclooxygenase in biology and disease. Faseb j 12: 1063-73.

Eagling VA, Howe JL, Barry MJ *et al* (1994) The metabolism of zidovudine by human liver microsomes in vitro: formation of 3'-amino-3'-deoxythymidine. Biochem Pharmacol 48: 267-76.

Elhasid R, Krivoy N, Rowe JM *et al* (2010) Influence of glutathione S-transferase A1, P1, M1, T1 polymorphisms on oral busulfan pharmacokinetics in children with congenital hemoglobinopathies undergoing hematopoietic stem cell transplantation. Pediatr Blood Cancer 55: 1172-9.

Engel G, Hofmann U, Heidemann H *et al* (1996) Antipyrine as a probe for human oxidative drug metabolism: identification of the cytochrome P450 enzymes catalyzing 4-hydroxyantipyrine, 3-hydroxymethylantipyrine, and norantipyrine formation. Clin Pharmacol Ther 59: 613-23.

Evans WE & Relling MV (2004) Moving towards individualized medicine with pharmacogenomics. Nature 429: 464-8.

Fang J, Coutts RT, Mckenna KF *et al* (1998) Elucidation of individual cytochrome P450 enzymes involved in the metabolism of clozapine. Naunyn Schmiedebergs Arch Pharmacol 358: 592-9.

Fda (2012) Healthy People Co. Issues a Voluntary Recall of Specific Lots of the Dietary Supplements Found to Contain Undeclared Drug Ingredients [Online]. In: U.S. Food and Drugs Administration. Available via http://www.fda.gov/safety/recalls/ucm290416.htm. [Cited 26/01/2014.

Fda (2014) Q&A on Dietary Supplements [Online]. In: U.S. Food and Drugs Administration. Available via http://www.fda.gov/Food/DietarySupplements/QADietarySupplements/default.htm#what_is. [Cited 04/04/2014.

Findlay VJ, Townsend DM, Saavedra JE *et al* (2004) Tumor cell responses to a novel glutathione S-transferase-activated nitric oxide-releasing prodrug. Mol Pharmacol 65: 1070-9.

Foti RS, Pearson JT, Rock DA *et al* (2009) In vitro inhibition of multiple cytochrome P450 isoforms by xanthone derivatives from mangosteen extract. Drug Metab Dispos 37: 1848-55.

Foti RS & Wahlstrom JL (2008) The role of dietary supplements in cytochrome P450-mediated drug interactions. Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas 7: 66 - 84.

Frangogiannis NG, Smith CW & Entman ML (2002) The inflammatory response in myocardial infarction. Cardiovasc Res 53: 31-47.

Fung HB, Kirschenbaum HL & Hameed R (2000) Amprenavir: a new human immunodeficiency virus type 1 protease inhibitor. Clin Ther 22: 549-72.

Garcia-Martin E, Martinez C, Tabares B *et al* (2004) Interindividual variability in ibuprofen pharmacokinetics is related to interaction of cytochrome P450 2C8 and 2C9 amino acid polymorphisms. Clin Pharmacol Ther 76: 119-27.

Garrity AR, Morton GA & Morton JC. 2004. *Nutraceutical mangosteen composition*. US patent application 10/283,600.

Ghahramani P, Ellis SW, Lennard MS *et al* (1997) Cytochromes P450 mediating the N-demethylation of amitriptyline. Br J Clin Pharmacol 43: 137-44.

Ghosh S, May MJ & Kopp EB (1998) NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol 16: 225-60.

Gianluigi Zaza, Meyling Cheok, Natalia Krynetskaia *et al* (2010) Thiopurine pathway. Pharmacogenetics and genomics 20(9): 573–574.

Goldstein JA, Faletto MB, Romkes-Sparks M *et al* (1994) Evidence that CYP2C19 is the major (S)-mephenytoin 4'-hydroxylase in humans. Biochemistry 33: 1743-52.

Gross SS & Wolin MS (1995) Nitric oxide: pathophysiological mechanisms. Annu Rev Physiol 57: 737-69.

Guitton J, Buronfosse T, Desage M *et al* (1998) Possible involvement of multiple human cytochrome P450 isoforms in the liver metabolism of propofol. Br J Anaesth 80: 788-95.

Gutierrez-Orozco F, Chitchumroonchokchai C, Lesinski GB *et al* (2013) alpha-Mangostin: anti-inflammatory activity and metabolism by human cells. J Agric Food Chem 61: 3891-900.

Gutierrez-Orozco F & Failla ML (2013) Biological activities and bioavailability of mangosteen xanthones: a critical review of the current evidence. Nutrients 5: 3163-83.

Gutkin DW & Shurin MR (2014) Clinical evaluation of systemic and local immune responses in cancer: time for integration. Cancer Immunol Immunother 63: 45-57.

Hallberg P, Karlsson J, Kurland L *et al* (2002) The CYP2C9 genotype predicts the blood pressure response to irbesartan: results from the Swedish Irbesartan Left Ventricular Hypertrophy Investigation vs Atenolol (SILVHIA) trial. J Hypertens 20: 2089-93.

Hamilton DS, Zhang X, Ding Z *et al* (2003) Mechanism of the glutathione transferase-catalyzed conversion of antitumor 2-crotonyloxymethyl-2-cycloalkenones to GSH adducts. J Am Chem Soc 125: 15049-58.

Hanahan D & Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144: 646-74.

Hayes JD, Flanagan JU & Jowsey IR (2005) Glutathione transferases. Annu Rev Pharmacol Toxicol 45: 51-88.

Hayes JD & Pulford DJ (1995) The Glutathione S-Transferase Supergene Family: Regulation of GST and the Contribution of the Isoenzymes to Cancer Chemoprotection and Drug Resistance Part I. Critical Reviews in Biochemistry and Molecular Biology 30: 445-520.

He K, Talaat RE, Pool WF *et al* (2004) Metabolic activation of troglitazone: identification of a reactive metabolite and mechanisms involved. Drug Metab Dispos 32: 639-46.

Hijazi Y & Boulieu R (2002) Contribution of CYP3A4, CYP2B6, and CYP2C9 isoforms to N-demethylation of ketamine in human liver microsomes. Drug Metab Dispos 30: 853-8.

Ibrahim MY, Hashim NM, Mohan S *et al* (2013) α-Mangostin from Cratoxylum arborescens: An in vitro and in vivo Toxicological Evaluation. Arabian Journal of Chemistry.

Ichiki H, Miura T, Kubo M *et al* (1998) New antidiabetic compounds, mangiferin and its glucoside. Biol Pharm Bull 21: 1389-90.

Itoh T, Ohguchi K, Iinuma M *et al* (2008) Inhibitory effect of xanthones isolated from the pericarp of Garcinia mangostana L. on rat basophilic leukemia RBL-2H3 cell degranulation. Bioorg Med Chem 16: 4500-8.

Jaakkola T, Backman JT, Neuvonen M *et al* (2006) Montelukast and zafirlukast do not affect the pharmacokinetics of the CYP2C8 substrate pioglitazone. Eur J Clin Pharmacol 62: 503-9.

Johnson JJ, Petiwala SM, Syed DN *et al* (2012) α-Mangostin, a xanthone from mangosteen fruit, promotes cell cycle arrest in prostate cancer and decreases xenograft tumor growth. Carcinogenesis 33: 413-419.

Jordan SA, Cunningham DG & Marles RJ (2010) Assessment of herbal medicinal products: challenges, and opportunities to increase the knowledge base for safety assessment. Toxicol Appl Pharmacol 243: 198-216.

Jung H-A, Su B-N, Keller WJ *et al* (2006) Antioxidant Xanthones from the Pericarp of Garcinia mangostana (Mangosteen). Journal of Agricultural and Food Chemistry 54: 2077-2082.

Kalra K, Jarmal G & Mishra N (2009) Drugs impact on CYP-450 enzyme family: A pharmacogenetical study of response variation. Indian J Hum Genet 15: 78-83.

Kaomongkolgit R, Jamdee K & Chaisomboon N (2009) Antifungal activity of alpha-mangostin against Candida albicans. J Oral Sci 51: 401-6.

Kendall A & Dowsett M (2006) Novel concepts for the chemoprevention of breast cancer through aromatase inhibition. Endocr Relat Cancer 13: 827-37.

Kerdpin O, Elliot DJ, Boye SL *et al* (2004) Differential contribution of active site residues in substrate recognition sites 1 and 5 to cytochrome P450 2C8 substrate selectivity and regioselectivity. Biochemistry 43: 7834-42.

Knox C, Law V, Jewison T *et al* (2011) DrugBank 3.0: a comprehensive resource for 'omics' research on drugs. Nucleic Acids Res 39: D1035-41.

Kosem N, Han Y-H & Moongkarndi P (2007) Antioxidant and Cytoprotective Activities of Methanolic Extract from Garcinia mangostana Hulls. Science Asia 33: 283-292.

Krajarng A, Nakamura Y, Suksamrarn S *et al* (2011) α-Mangostin Induces Apoptosis in Human Chondrosarcoma Cells through Downregulation of ERK/JNK and Akt Signaling Pathway. Journal of Agricultural and Food Chemistry 59: 5746-5754.

Kristensen K, Christensen CB, Christrup LL *et al* (1996) The mu1 and mu2 opioid receptor binding of ketobemidone, norketobemidone and 3-dimethylamino-1,1-diphenylbutene. Pharmacol Toxicol 79: 103-4.

Kumar GN, Dubberke E, Rodrigues AD *et al* (1997) Identification of cytochromes P450 involved in the human liver microsomal metabolism of the thromboxane A2 inhibitor seratrodast (ABT-001). Drug Metab Dispos 25: 110-5.

Kumar V, Abbas AK, Fausto N *et al* (2012) Robbins Basic Pathology, Elsevier Health Sciences.

Kurata N, Nishimura Y, Iwase M *et al* (1998) Trimethadione metabolism by human liver cytochrome P450: evidence for the involvement of CYP2E1. Xenobiotica 28: 1041-7.

Kurose H, Shibata MA, Iinuma M *et al* (2012) Alterations in cell cycle and induction of apoptotic cell death in breast cancer cells treated with alpha-mangostin extracted from mangosteen pericarp. J Biomed Biotechnol 2012: 672428.

Kusche J, Bieganski T, Hesterberg R *et al* (1980) The influence of carcinoma growth on diamine oxidase activity in human gastrointestinal tract. Agents Actions 10: 110-3.

Lala PK & Chakraborty C (2001) Role of nitric oxide in carcinogenesis and tumour progression. Lancet Oncol 2: 149-56.

Lala PK & Orucevic A (1998) Role of nitric oxide in tumor progression: lessons from experimental tumors. Cancer Metastasis Rev 17: 91-106.

Lawenda BD, Kelly KM, Ladas EJ *et al* (2008) Should supplemental antioxidant administration be avoided during chemotherapy and radiation therapy? J Natl Cancer Inst 100: 773-83.

Leão M, Gomes S, Pedraza-Chaverri J *et al* (2013) Alpha-mangostin and gambogic acid as potential inhibitors of the p53-MDM2 interaction revealed by a yeast approach. J Nat Prod 76: 774-8.

Lesourd B (2002) [Malnutrition and immunity]. Rev Med Interne 23 Suppl 2: 353s-359s.

Li Q & Verma IM (2002) NF-kappaB regulation in the immune system. Nat Rev Immunol 2: 725-34.

Lien S, Larsson AK & Mannervik B (2002) The polymorphic human glutathione transferase T1-1, the most efficient glutathione transferase in the denitrosation and inactivation of the anticancer drug 1,3-bis(2-chloroethyl)-1-nitrosourea. Biochem Pharmacol 63: 191-7.

Liu SH, Lee LT, Hu NY *et al* (2012) Effects of alpha-mangostin on the expression of anti-inflammatory genes in U937 cells. Chin Med 7: 19.

Liu Z, Antalek M, Nguyen L *et al* (2013) The effect of gartanin, a naturally occurring xanthone in mangosteen juice, on the mTOR pathway, autophagy, apoptosis, and the growth of human urinary bladder cancer cell lines. Nutr Cancer 65 Suppl 1: 68-77.

Lu AY (1998) Drug-metabolism research challenges in the new millennium: individual variability in drug therapy and drug safety. Drug Metab Dispos 26: 1217-22.

Maintz L & Novak N (2007) Histamine and histamine intolerance. Am J Clin Nutr 85: 1185-96.

Malinowski JM & Bolesta S (2000) Rosiglitazone in the treatment of type 2 diabetes mellitus: a critical review. Clin Ther 22: 1151-68; discussion 1149-50.

Mancy A, Antignac M, Minoletti C *et al* (1999) Diclofenac and its derivatives as tools for studying human cytochromes P450 active sites: particular efficiency and regioselectivity of P450 2Cs. Biochemistry 38: 14264-70.

Mannheimer B & Eliasson E (2010) Drug-drug interactions that reduce the formation of pharmacologically active metabolites: a poorly understood problem in clinical practice. J Intern Med 268: 540-8.

Mans DR, Lafleur MV, Westmijze EJ *et al* (1992) Reactions of glutathione with the catechol, the ortho-quinone and the semi-quinone free radical of etoposide. Consequences for DNA inactivation. Biochem Pharmacol 43: 1761-8.

Marcason W (2006) What are the facts and myths about mangosteen? J Am Diet Assoc 106: 986.

Marnett LJ (2009) The COXIB experience: a look in the rearview mirror. Annu Rev Pharmacol Toxicol 49: 265-90.

Marsh S, Mcleod H, Dolan E *et al* (2009) Platinum pathway. Pharmacogenet Genomics 19: 563-4.

Masferrer JL, Zweifel BS, Manning PT *et al* (1994) Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. Proc Natl Acad Sci U S A 91: 3228-32.

Matsumoto K, Akao Y, Kobayashi E *et al* (2003) Induction of apoptosis by xanthones from mangosteen in human leukemia cell lines. Journal of Natural Products 66: 1124-1127.

Matsumoto K, Akao Y, Ohguchi K *et al* (2005) Xanthones induce cell-cycle arrest and apoptosis in human colon cancer DLD-1 cells. Bioorg Med Chem 13: 6064-9.

Matzinger P (2002) The danger model: a renewed sense of self. Science 296: 301-5.

Mazimba O, Nana F, Kuete V *et al* (2013) 11 - Xanthones and Anthranoids from the Medicinal Plants of Africa. In*:* KUETE, V. (ed.) Medicinal Plant Research in Africa. Oxford: Elsevier.

Miura T, Ichiki H, Hashimoto I *et al* (2001a) Antidiabetic activity of a xanthone compound, mangiferin. Phytomedicine 8: 85-7.

Miura T, Ichiki H, Iwamoto N *et al* (2001b) Antidiabetic activity of the rhizoma of Anemarrhena asphodeloides and active components, mangiferin and its glucoside. Biol Pharm Bull 24: 1009-11.

Mocellin S, Bronte V & Nitti D (2007) Nitric oxide, a double edged sword in cancer biology: searching for therapeutic opportunities. Med Res Rev 27: 317-52.

Moongkarndi P, Kosem N, Kaslungka S *et al* (2004) Antiproliferation, antioxidation and induction of apoptosis by Garcinia mangostana (mangosteen) on SKBR3 human breast cancer cell line. Journal of Ethnopharmacology 90: 161-166.

Morale MC, L'episcopo F, Tirolo C *et al* (2008) Loss of aromatase cytochrome P450 function as a risk factor for Parkinson's disease? Brain Res Rev 57: 431-43.

Morgan AS, Sanderson PE, Borch RF *et al* (1998) Tumor efficacy and bone marrow-sparing properties of TER286, a cytotoxin activated by glutathione S-transferase. Cancer Res 58: 2568-75.

Moss RW (2007) Do antioxidants interfere with radiation therapy for cancer? Integr Cancer Ther 6: 281-92.

Nakagawa Y, Iinuma M, Naoe T *et al* (2007) Characterized mechanism of α-mangostin-induced cell death: Caspase-independent apoptosis with release of endonuclease-G from mitochondria and increased miR-143 expression in human colorectal cancer DLD-1 cells. Bioorganic and Medicinal Chemistry 15: 5620-5628.

Nakatani K, Atsumi M, Arakawa T *et al* (2002a) Inhibitions of histamine release and prostaglandin E2 synthesis by mangosteen, a Thai medicinal plant. Biol Pharm Bull 25: 1137-41.

Nakatani K, Nakahata N, Arakawa T *et al* (2002b) Inhibition of cyclooxygenase and prostaglandin E2 synthesis by gamma-mangostin, a xanthone derivative in mangosteen, in C6 rat glioma cells. Biochem Pharmacol 63: 73-9.

Nakatani K, Yamakuni T, Kondo N *et al* (2004) gamma-Mangostin inhibits inhibitor-kappaB kinase activity and decreases lipopolysaccharide-induced cyclooxygenase-2 gene expression in C6 rat glioma cells. Mol Pharmacol 66: 667-74.

Narang PK, Crouthamel WG, Carliner NH *et al* (1978) Lidocaine and its active metabolites. Clin Pharmacol Ther 24: 654-62.

Nekhayeva IA, Nanovskaya TN, Deshmukh SV *et al* (2005) Bidirectional transfer of methadone across human placenta. Biochem Pharmacol 69: 187-97.

Nielsen TL, Rasmussen BB, Flinois JP *et al* (1999) In vitro metabolism of quinidine: the (3S)-3-hydroxylation of quinidine is a specific marker reaction for cytochrome P-4503A4 activity in human liver microsomes. J Pharmacol Exp Ther 289: 31-7.

Oakley A (2011) Glutathione transferases: a structural perspective. Drug Metab Rev 43: 138-51.

Obolskiy D, Pischel I, Siriwatanametanon N *et al* (2009) Garcinia mangostana L.: a phytochemical and pharmacological review. Phytother Res 23: 1047-65.

Olesen OV & Linnet K (1997) Metabolism of the tricyclic antidepressant amitriptyline by cDNA-expressed human cytochrome P450 enzymes. Pharmacology 55: 235-43.

Olesen OV & Linnet K (2000) Identification of the human cytochrome P450 isoforms mediating in vitro N-dealkylation of perphenazine. Br J Clin Pharmacol 50: 563-71.

Ono S, Hatanaka T, Miyazawa S *et al* (1996) Human liver microsomal diazepam metabolism using cDNA-expressed cytochrome P450s: role of CYP2B6, 2C19 and the 3A subfamily. Xenobiotica 26: 1155-66.

Paakki P, Kirkinen P, Helin H *et al* (2000) Antepartum glucocorticoid therapy suppresses human placental xenobiotic and steroid metabolizing enzymes. Placenta 21: 241-6.

Parker LJ, Ciccone S, Italiano LC *et al* (2008) The anti-cancer drug chlorambucil as a substrate for the human polymorphic enzyme glutathione transferase P1-1: kinetic properties and crystallographic characterisation of allelic variants. J Mol Biol 380: 131-44.

Pearce RE, Rodrigues AD, Goldstein JA *et al* (1996) Identification of the human P450 enzymes involved in lansoprazole metabolism. J Pharmacol Exp Ther 277: 805-16.

Pedraza-Chaverri J, Cárdenas-Rodríguez N, Orozco-Ibarra M *et al* (2008) Medicinal properties of mangosteen (Garcinia mangostana). Food and Chemical Toxicology 46: 3227-3239.

Pedro M, Cerqueira F, Sousa ME *et al* (2002) Xanthones as inhibitors of growth of human cancer cell lines and their effects on the proliferation of human lymphocytes in vitro. Bioorganic & medicinal chemistry 10: 3725-30.

Peklak-Scott C, Townsend AJ & Morrow CS (2005) Dynamics of glutathione conjugation and conjugate efflux in detoxification of the carcinogen, 4-nitroquinoline 1-oxide: contributions of glutathione, glutathione S-transferase, and MRP1. Biochemistry 44: 4426-33.

Perini JA, Vianna-Jorge R, Brogliato AR *et al* (2005) Influence of CYP2C9 genotypes on the pharmacokinetics and pharmacodynamics of piroxicam. Clin Pharmacol Ther 78: 362-9.

Peters U, Preisler-Adams S, Hebeisen A *et al* (2000) Glutathione S-transferase genetic polymorphisms and individual sensitivity to the ototoxic effect of cisplatin. Anticancer Drugs 11: 639-43.

Pinto M & Castanheiro R (2009) Natural Prenylated Xanthones: Chemistry and Biological Activities. In*:* BRAHMACHARI, G. (ed.) Natural Products: Chemistry, Biochemistry and Pharmacology. Oxford, UK: Alpha science International.

Pinto MM, Sousa ME & Nascimento MS (2005) Xanthone derivatives: new insights in biological activities. Curr Med Chem 12: 2517-38.

Preissner S, Kroll K, Dunkel M *et al* (2010) SuperCYP: a comprehensive database on Cytochrome P450 enzymes including a tool for analysis of CYP-drug interactions. Nucleic Acids Res 38: D237-43.

Raithel M, Ulrich P, Hochberger J *et al* (1998) Measurement of gut diamine oxidase activity. Diamine oxidase as a new biologic marker of colorectal proliferation? Ann N Y Acad Sci 859: 262-6.

Ramana CV, Chatterjee-Kishore M, Nguyen H *et al* (2000) Complex roles of Stat1 in regulating gene expression. Oncogene 19: 2619-27.

Raucy JL, Lasker JM, Lieber CS *et al* (1989) Acetaminophen activation by human liver cytochromes P450IIE1 and P450IA2. Arch Biochem Biophys 271: 270-83.

Reilly CA, Ehlhardt WJ, Jackson DA *et al* (2003) Metabolism of capsaicin by cytochrome P450 produces novel dehydrogenated metabolites and decreases cytotoxicity to lung and liver cells. Chem Res Toxicol 16: 336-49.

Relling MV, Aoyama T, Gonzalez FJ *et al* (1990) Tolbutamide and mephenytoin hydroxylation by human cytochrome P450s in the CYP2C subfamily. J Pharmacol Exp Ther 252: 442-7.

Rendic S (2002) Summary of information on human CYP enzymes: human P450 metabolism data. Drug Metab Rev 34: 83-448.

Rettie AE & Tai G (2006) The pharmocogenomics of warfarin: closing in on personalized medicine. Mol Interv 6: 223-7.

Rostami-Hodjegan A, Amin AM, Spencer EP *et al* (2004) Influence of dose, cigarette smoking, age, sex, and metabolic activity on plasma clozapine concentrations: a predictive model and nomograms to aid clozapine dose adjustment and to assess compliance in individual patients. J Clin Psychopharmacol 24: 70-8.

Rouzer CA & Marnett LJ (2009) Cyclooxygenases: structural and functional insights. J Lipid Res 50 Suppl: S29-34.

Rozman B (2002) Clinical pharmacokinetics of leflunomide. Clin Pharmacokinet 41: 421-30.

Sahoo N & Manchikanti P (2013) Herbal drug regulation and commercialization: an Indian industry perspective. J Altern Complement Med 19: 957-63.

Sakagami Y, Iinuma M, Piyasena KG *et al* (2005) Antibacterial activity of alpha-mangostin against vancomycin resistant Enterococci (VRE) and synergism with antibiotics. Phytomedicine 12: 203-8.

Salonen JS, Nyman L, Boobis AR *et al* (2003) Comparative studies on the cytochrome p450-associated metabolism and interaction potential of selegiline between human liver-derived in vitro systems. Drug Metab Dispos 31: 1093-102.

Sampath PD & Vijayaragavan K (2008) Ameliorative prospective of alpha-mangostin, a xanthone derivative from Garcinia mangostana against beta-adrenergic cathecolamine-induced myocardial toxicity and anomalous cardiac TNF-alpha and COX-2 expressions in rats. Exp Toxicol Pathol 60: 357-64.

Scripture CD & Pieper JA (2001) Clinical pharmacokinetics of fluvastatin. Clin Pharmacokinet 40: 263-81.

Shan T, Ma Q, Guo K *et al* (2011) Xanthones from mangosteen extracts as natural chemopreventive agents: potential anticancer drugs. Curr Mol Med 11: 666-77.

Shankaranarayan D, Gopalakrishnan C & Kameswaran L (1979) Pharmacological profile of mangostin and its derivatives. Arch Int Pharmacodyn Ther 239: 257-69.

Shi S & Klotz U (2012) Drug interactions with herbal medicines. Clin Pharmacokinet 51: 77-104.

Shou M, Lu T, Krausz KW *et al* (2000) Use of inhibitory monoclonal antibodies to assess the contribution of cytochromes P450 to human drug metabolism. Eur J Pharmacol 394: 199-209.

Silberzahn P, Gaillard JL, Quincey D *et al* (1988) Aromatization of testosterone and 19-nortestosterone by a single enzyme from equine testicular microsomes. Differences from human placental aromatase. J Steroid Biochem 29: 119-25.

Simpson ER, Mahendroo MS, Means GD *et al* (1994) Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr Rev 15: 342-55.

Singh RP, Waldron RT & Hahn BH (2012) Genes, tolerance and systemic autoimmunity. Autoimmun Rev 11: 664-9.

Srinivasan V, Maestroni GJ, Cardinali DP *et al* (2005) Melatonin, immune function and aging. Immun Ageing 2: 17.

Srivastava PK, Yun CH, Beaune PH *et al* (1991) Separation of human liver microsomal tolbutamide hydroxylase and (S)-mephenytoin 4'-hydroxylase cytochrome P-450 enzymes. Mol Pharmacol 40: 69-79.

Stanner SA, Hughes J, Kelly CN *et al* (2004) A review of the epidemiological evidence for the 'antioxidant hypothesis'. Public Health Nutr 7: 407-22.

Stormer E, Von Moltke LL, Shader RI *et al* (2000) Metabolism of the antidepressant mirtazapine in vitro: contribution of cytochromes P-450 1A2, 2D6, and 3A4. Drug Metab Dispos 28: 1168-75.

Suksamrarn S, Komutiban O, Ratananukul P *et al* (2006) Cytotoxic prenylated xanthones from the young fruit of Garcinia mangostana. Chemical and Pharmaceutical Bulletin 54: 301-305.

Suksamrarn S, Suwannapoch N, Phakhodee W *et al* (2003) Antimycobacterial activity of prenylated xanthones from the fruits of Garcinia mangostana. Chem Pharm Bull (Tokyo) 51: 857-9.

Sutton D, Butler AM, Nadin L *et al* (1997) Role of CYP3A4 in human hepatic diltiazem N-demethylation: inhibition of CYP3A4 activity by oxidized diltiazem metabolites. J Pharmacol Exp Ther 282: 294-300.

Syljuasen RG, Krolewski B & Little JB (1999) Loss of normal G1 checkpoint control is an early step in carcinogenesis, independent of p53 status. Cancer Res 59: 1008-14.

Tanaka E, Kurata N & Yasuhara H (2003) Involvement of cytochrome P450 2C9, 2E1 and 3A4 in trimethadione N-demethylation in human microsomes. J Clin Pharm Ther 28: 493-6.

Tang YP, Li PG, Kondo M *et al* (2009) Effect of a mangosteen dietary supplement on human immune function: a randomized, double-blind, placebo-controlled trial. J Med Food 12: 755-63.

Teixeira M, Cerqueira F, Barbosa CM *et al* (2005) Improvement of the inhibitory effect of xanthones on NO production by encapsulation in PLGA nanocapsules. J Drug Target 13: 129-35.

Tew KD (2005) TLK-286: a novel glutathione S-transferase-activated prodrug. Expert Opin Investig Drugs 14: 1047-54.

Tewtrakul S, Wattanapiromsakul C & Mahabusarakam W (2009) Effects of compounds from Garcinia mangostana on inflammatory mediators in RAW264.7 macrophage cells. J Ethnopharmacol 121: 379-82.

Tracy TS, Korzekwa KR, Gonzalez FJ *et al* (1999) Cytochrome P450 isoforms involved in metabolism of the enantiomers of verapamil and norverapamil. Br J Clin Pharmacol 47: 545-52.

Turck D, Roth W & Busch U (1996) A review of the clinical pharmacokinetics of meloxicam. Br J Rheumatol 35 Suppl 1: 13-6.

Uetrecht JP & Trager W (2007) Drug Metabolism: Chemical and Enzymatic Aspects, Taylor & Francis.

Ufer M, Svensson JO, Krausz KW *et al* (2004) Identification of cytochromes P450 2C9 and 3A4 as the major catalysts of phenprocoumon hydroxylation in vitro. Eur J Clin Pharmacol 60: 173-82.

Valko M, Leibfritz D, Moncol J *et al* (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39: 44-84.

Vassord C, Lapoumeroulie C, Koumaravelou K *et al* (2008) Endothelial cells do not express GSTA1: potential relevance to busulfan-mediated endothelial damage during haematopoietic stem cell transplantation. Eur J Haematol 80: 299-302.

Vickers AE, Sinclair JR, Zollinger M *et al* (1999) Multiple cytochrome P-450s involved in the metabolism of terbinafine suggest a limited potential for drug-drug interactions. Drug Metab Dispos 27: 1029-38.

Von Moltke LL, Greenblatt DJ, Grassi JM *et al* (1998) Multiple human cytochromes contribute to biotransformation of dextromethorphan in-vitro: role of CYP2C9, CYP2C19, CYP2D6, and CYP3A. J Pharm Pharmacol 50: 997-1004.

Vormfelde SV, Engelhardt S, Zirk A *et al* (2004) CYP2C9 polymorphisms and the interindividual variability in pharmacokinetics and pharmacodynamics of the loop diuretic drug torsemide. Clin Pharmacol Ther 76: 557-66.

Wada T & Penninger JM (2004) Mitogen-activated protein kinases in apoptosis regulation. Oncogene 23: 2838-49.

Wang JJ, Sanderson BJS & Zhang W (2011) Cytotoxic effect of xanthones from pericarp of the tropical fruit mangosteen (Garcinia mangostana Linn.) on human melanoma cells. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 49: 2385-91.

Wang JJ, Zhang W & Sanderson BJ (2013) Altered mRNA expression related to the apoptotic effect of three xanthones on human melanoma SK-MEL-28 cell line. Biomed Res Int 2013: 715603.

Watanapokasin R, Jarinthanan F, Jerusalmi A *et al* (2010) Potential of Xanthones from Tropical Fruit Mangosteen as Anti-cancer Agents: Caspase-Dependent Apoptosis Induction In Vitro and in Mice. Applied Biochemistry and Biotechnology 162: 1080-1094.

Weecharangsan W, Opanasopit P, Sukma M *et al* (2006) Antioxidative and neuroprotective activities of extracts from the fruit hull of mangosteen (Garcinia mangostana Linn.). Med Princ Pract 15: 281-7.

Wink DA, Kasprzak KS, Maragos CM *et al* (1991) DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. Science 254: 1001-3.

Wink DA, Vodovotz Y, Laval J *et al* (1998) The multifaceted roles of nitric oxide in cancer. Carcinogenesis 19: 711-21.

Winter HR, Wang Y & Unadkat JD (2000) CYP2C8/9 mediate dapsone N-hydroxylation at clinical concentrations of dapsone. Drug Metab Dispos 28: 865-8.

Wong LP & Klemmer PJ (2008) Severe lactic acidosis associated with juice of the mangosteen fruit Garcinia mangostana. Am J Kidney Dis 51: 829-33.

Yamazaki H, Inoue K, Hashimoto M *et al* (1999a) Roles of CYP2A6 and CYP2B6 in nicotine C-oxidation by human liver microsomes. Arch Toxicol 73: 65-70.

Yamazaki H, Shaw PM, Guengerich FP *et al* (1998) Roles of cytochromes P450 1A2 and 3A4 in the oxidation of estradiol and estrone in human liver microsomes. Chem Res Toxicol 11: 659-65.

Yamazaki H, Shibata A, Suzuki M *et al* (1999b) Oxidation of troglitazone to a quinone-type metabolite catalyzed by cytochrome P-450 2C8 and P-450 3A4 in human liver microsomes. Drug Metab Dispos 27: 1260-6.

Yamazaki H & Shimada T (1997) Progesterone and testosterone hydroxylation by cytochromes P450 2C19, 2C9, and 3A4 in human liver microsomes. Arch Biochem Biophys 346: 161-9.

Yang TJ, Shou M, Korzekwa KR *et al* (1998) Role of cDNA-expressed human cytochromes P450 in the metabolism of diazepam. Biochem Pharmacol 55: 889-96.

Yasar U, Annas A, Svensson JO *et al* (2005) Ketobemidone is a substrate for cytochrome P4502C9 and 3A4, but not for P-glycoprotein. Xenobiotica 35: 785-96.

Yasuda H (2008) Solid tumor physiology and hypoxia-induced chemo/radio-resistance: novel strategy for cancer therapy: nitric oxide donor as a therapeutic enhancer. Nitric Oxide 19: 205-16.

Yoo J-H, Kang K, Jho EH *et al* (2011) α- and γ-Mangostin inhibit the proliferation of colon cancer cells via β-catenin gene regulation in Wnt/cGMP signalling. Food Chemistry 129: 1559-1566.

Zhao J, Leemann T & Dayer P (1992) In vitro oxidation of oxicam NSAIDS by a human liver cytochrome P450. Life Sci 51: 575-81.

Zhou SF, Zhou ZW, Yang LP *et al* (2009) Substrates, inducers, inhibitors and structure-activity relationships of human Cytochrome P450 2C9 and implications in drug development. Curr Med Chem 16: 3480-675.

Ziche M & Morbidelli L (2000) Nitric oxide and angiogenesis. J Neurooncol 50: 139-48.

Paper 2 (draft): "Alpha-mangostin antitumor activity: cytotoxicity and influence on the immune system microenvironment"

Abstract

Alpha-mangostin is known to interfere with multiple pathways of carcinogenesis. Therefore, it has been proposed as prospective cancer drug candidate. In melanoma, alpha-mangostin antitumor effect may be increased by the immunomodulation of tumor microenvironment, since melanocytic tumors are highly immunogenic.

The main gold of this study was evaluate alpha-mangostin (i) cytotoxic effect on A375-C5 melanoma cell line and (ii) it interference with the immune macrophages induced microenvironment of the tumor.

This xanthone showed inhibition of A375-C5 melanoma growth (GI_{50} = 11.3 ± 0.9 μM). Concerning the direct impact of alpha-mangostin on macrophages function, it was observed an inhibition of nitric oxide production by RAW 264.7 murine macrophages (IC_{50} = 13.8 ± 0.7 μM) and decreased the concentration of IL-1β and TGF-β1 and stimulated TNF-α expression by THP-1 human macrophages. In order to complement the described effect of α-MG on the immune microenvironment of melanoma, it was also study in proliferation and cytokines production by human lymphocytes. Alpha-mangostin strongly inhibited PHA-stimulated human mononuclear cells proliferation (IC_{50} = 9.2 ± 0.3 μM) but not interfering for IC_{50} for IL-1β, TNF-α, IL-10 and TGF-β1.

In conclusion this study proved that alpha-mangostin interferes with cytokine and NO production by activated macrophages leading to the evidence that the xanthone interferes with immune microenvironment of the tumor.

Introduction

Melanoma is a melanocytes' malignant tumor that metastasizes very early in the disease process, causing elevated mortality rates. According to the Oncologic register of Portugal (2007), the world incidence of melanoma of the skin is about 4,5 cases for 100000 habitants (RORENO 2013) and, although it is a relative rare kind of cancer, it contributes for about 80% of the deaths caused by cutaneous cancer (Kuphal and Bosserhoff 2009; Gast *et al.* 2011; Raaijmakers *et al.* 2013). The tumorigenesis of this cancer requires a multistep process, in spite of alterations in immune system has a crucial involvement, namely the immunosuppressive and pro-tumor character of tumor microenvironment (Hussein 2004; Chen *et al.* 2011). Among inflammatory cells,

macrophages are of pivotal importance in malignancy development, being specifically referred to as tumor-associated macrophages (TAMs) (Chen *et al.* 2011). TAMs are the most abundant leukocytes in the melanoma and their presence represent a poor prognostic (Brocker *et al.* 1988; Bernengo *et al.* 2000; Makitie *et al.* 2001; Varney *et al.* 2005), due to induction of an immunosuppressive microenvironment that, among other consequences, inhibits tumor-specific $CD8^+$ T cell-mediated cytotoxicity (Kono *et al.* 1996; Wang *et al.* 2012c).

The growing understanding of immune involvement in tumorigenesis, contributed to therapies aiming immunologic targets (Dranoff 2009; Raaijmakers *et al.* 2013). However some of these therapies are only effective in a brief period of time while others have considerable side effects (Hodi *et al.* 2010; Solit and Rosen 2011; Graziani *et al.* 2012). Obviously, it is important to find new therapies for melanoma that target multiple biochemical pathways, in order to avoid tumor escape mechanisms (Smalley *et al.* 2006).

Prenylated xanthones, including alpha-mangostin (α-MG) (**Figure 9**), the most studied and abundant xanthone present on mangosteen pericarp (*Garcinia mangostana* Linn), have been associated to multiple benefits in several pathological conditions. In cancer, its effect is through the alteration of a significant number of physiological pathways, comprising the influence in immune system (Obolskiy *et al.* 2009; Shan *et al.* 2011; Gutierrez-Orozco and Failla 2013). Beside the previous reports of α-MG effect in immune functions, as far as we know the interference of this xanthone in the immune system microenvironment of melanoma has never been studied.

Recently, a number of evidences suggested that α-MG has a potent antitumor effect against melanoma. These reports evaluated the effect of the compound in different melanoma cell lines (B16-F10 murine cell line, SK-MEL-28 human cell line) and described induction of apoptosis, cell cycle arrest and differentiation, and inhibition of both cell proliferation and metastasis (Wang *et al.* 2011; Wang *et al.* 2012a; Wang *et al.* 2012b; Wang *et al.* 2013). During the development of this study, Beninati *et al* reported some experiments of α-MG effect on the A375 cell line (Beninati *et al.* 2014).

In spite of these insights, and as far as we know, no studies on α-MG-dependent modulation of the immune microenvironment and its effect on melanoma growth were performed.

Due to the importance of immune targets in melanoma therapies, the aim of the present work is to study α-MG effects on the immune system in order to potentiate its antitumor effect on A375-C5 melanoma cell line.

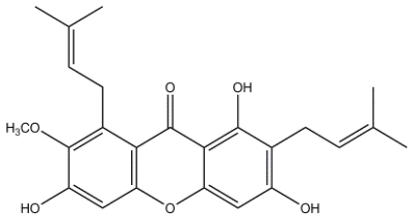


Figure 9: alpha-Mangostin

Material and Methods

Chemicals and reagents

Reagents used in cell culture, including RPMI-1640, DMEM medium, and fetal bovine serum (FBS) were purchased from Gibco® Invitrogen Co. (Barcelona, Spain), 2-mercaptoethanol for synthesis was obtained from Merck (Whitehouse Station, NJ, USA) and Glutamine cell culture grade and Dimethyl sulfoxide (DMSO) from Applichem (Darmstadt, Germany). N.N-Dimetilformamida (DMF) was acquiring from Spectramol Science Incorporated.

Reagents used in SRB assay as Trichloroacetic acid (TCA), Acetic acid glacial 99-100% and Trizma base were purchased from Prolabo (Oeiras, Portugal), CHEM-LAB (Zedelgem, Belgium) and Frilabo (Maia, Portugal), respectively.

IL-1β, IL-10, TNF-α and TGF-β1 ELISA Ready-Set-Go Kits were acquired from eBioscience (San Diego, CA, USA).

All other chemicals of analytical grade used in the experiments and unless otherwise indicated were purchased from Sigma-Aldrich® (St. Louis, MO, USA).

Xanthones

Alpha-mangostin (α-MG) was obtained from Sigma-Aldrich® (ref.M3824) and stock solution kept at -20ºC in DMSO. Just prior each assay, stock solution was diluted in appropriate complete medium to the maximum concentration to test and 1:2 dilutions were serially prepared.

Cell lines

A375-C5 human malignant melanoma cell line and RAW 264.7 mouse macrophage cell line are part of CEQUIMED cell culture collection. THP-1 human monocyte cell line was a courtesy of Rui Appelberg.

A375-C5 was routinely maintained in 25 cm^2 flasks containing RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1 µL mL^{-1} Gentamycin at 37ºC in a humidified incubator with 5% CO_2. For the THP-1 cell line the culture medium just

described was also supplemented with 0.05 mM 2-mercaptoethanol. RAW 264.7 cell line was maintained in 75 cm^2 flasks containing DMEM medium with 10% FBS and gentamycin, in the same culture conditions.

Cell lines were sub-cultured every 2 or 3 days by trypsinization (A375-C5), replacement of the medium (THP-1) or scraping (RAW 264.7) and used for each experiment when cells were in exponential growth.

Human mononuclear cells were isolated from peripheral blood of healthy volunteers by Histopaque-1077 density centrifugation according to manufactures' instructions. Informed consent was obtained from each volunteer. Cells were ressuspended in RPMI medium supplemented with 10% FBS and 1 µL mL^{-1} gentamycin.

NO production assay

The determination of nitric oxide (NO) production by RAW 264.7 (1 X 10^6 cells mL^{-1}) after treatment and stimulation with 1.5 µg mL^{-1} LPS was quantified by Griess assay as previous described by our group (Teixeira *et al.* 2005; Cerqueira *et al.* 2008). *N*-nitro-L-arginine methyl ester (L-NAME), an inhibitor of inductive nitric oxide synthase (iNOS) activity and Dexamethasone, an inhibitor of iNOS expression, were used as positive controls.

Since inhibition of NO production could be a consequence of an inhibition of iNOS expression and/or activity, it was necessary to investigate α-MG mechanism. For that, the compound was also added 6h and 14h after RAW 264.7 macrophages stimulation with LPS (Cerqueira *et al.* 2008).

NO Scavenging assay

To discard NO scavenging effect by xanthone, nitrite was chemically generated using sodium nitroprusside as previously described. Nitrite was quantified by Griess assay (Teixeira *et al.* 2005; Cerqueira *et al.* 2008).

Human mononuclear cells MTT-proliferation assay

Human mononuclear cells isolated from peripheral blood were plated (2-3 X 10^6 cell mL^{-1}), treated with serial dilutions of xanthone, stimulated with 10 µg mL^{-1} phytohemaglutinin (PHA) and incubated for 96h. After incubation, the MTT-proliferation assay was performed and absorbance measured at 550 nm. Cyclosporin A was used as positive control (Pedro *et al.* 2002; Cerqueira *et al.* 2003; Teixeira *et al.* 2005).

Cytokine quantification

Culture supernatants of differentiated THP-1 cells, stimulated and treated as previously described, were stored at -20ºC until cytokine analyses.

Levels of interleukin-1β (IL-1β), interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGFβ1) in culture supernatants were quantified by ELISA Ready-Set-Go Kits (eBioscience) according to manufacturers' instructions and measured at 510 nm in an ELISA reader (Stat Fax 3200, Awareness Technology) (Cerqueira *et al.* 2003).

The same cytokines were also evaluated in supernatants from peripheral mononuclear cells. Cells were stimulated with PHA, treated with tree concentrations of α-MG and incubated for 48h. Then, supernatants were collected and stored until analysis (Cerqueira *et al.* 2003; Lou *et al.* 2013).

Sulphorrodamine B (SRB) growth tumor inhibition assay

α-MG effect on the growth of A375-C5 human melanoma cell line (7,5 X 10^4 cells mL^{-1}) was evaluated according to the method adopted by the National Cancer Institute (NCI, USA) (Monks *et al.* 1991), as already described by our group (Pedro *et al.* 2002; Gupta *et al.* 2008). Doxorubicin (1:10 dilutions) was used as positive control (Pedro *et al.* 2002; Gupta *et al.* 2008).

Antitumor effect of conditioned macrophage culture medium

After testing the cytotoxic effect of α-MG on A375-C5 melanoma cell line, the antitumor effect of conditioned macrophage culture medium was evaluated according to He *et al*, 2012. Briefly, THP-1 cell line was plated at 1 X 10^5 cells mL^{-1} and differentiated into THP-1 macrophages with 10 ng mL^{-1} PMA for 72h (He *et al.* 2012). Once differentiated, cells were washed twice with complete medium and left for another 24h incubation in order to obtain the resting state of macrophages (Chanput *et al.* 2012; Chanput *et al.* 2014). Then, they were stimulated with 100 µL of LPS solution (1 µg mL^{-1}) and treated with 100 µL α-MG (concentration below GI_{50}). Treated cell were incubated for 24h at 37ºC, 5% CO_2 in a humidified incubator (He *et al.* 2012). Plates were centrifuged and half of the volume of each well was transferred to A375-C5 adherent cell monolayers, previously plated as described for tumor growth inhibition assay procedure. After 48h incubation, SRB assay was performed, absorbance was measured and cell-growth inhibition determined (Pedro *et al.* 2002).

MTT-viability assay

Toxicity of α-MG on THP-1 cell line, human mononuclear cells and RAW 254.7 cell line was evaluated by MTT-viability assay (Pedro *et al.* 2002; Cerqueira *et al.* 2003; Teixeira *et al.* 2005).

Statistical analysis

Except otherwise stated, results are the mean ± SEM of at least three independent experiments, performed in duplicate. Statistical analysis was performed with SPSS for Windows (version 20.0). Statistical significance between groups was calculated by Mann-Whitney Test and it is considered significant for p values less than 0.05.

Ethics

Ethics approval was obtained by the Ethic Comity of University Fernando Pessoa.

Results

Effect on NO production by macrophages

The production of NO by RAW 264.7 was strongly inhibited by α-MG (IC_{50} = 13.8 ± 0.7 μM) in a dose-dependent manner (data not shown). Dexamethasone showed an IC_{50} of 4.3 ± 0.6 μM (**Table 2**). To exclude cell death, MTT assay was performed and exposed that viability was higher that 90% for IC_{50}, therefore, not responsible for the decrease in NO production (**Table 2**). Scavenging of NO, was not detected since the NO production in presence and absence of α-MG was similar (data not show). This finds, indicating that scavenging could not be responsible for the 50% reduction of NO production in RAW 264.7 cell line.

Table 2: Effect of α-MG on NO production by LPS-stimulated RAW 264.7 macrophages.

	NO inhibition (IC_{50})	Viability at IC_{50}
Alpha-mangostin	13.8 ± 0.7 μM	91.3 ± 3.7 %
L-Name	62.4 ± 7.8 μM	96.0 ± 2.5 %
Dexamethasone	4.3 ± 0.6 μM	100 %

Results are the mean ± SEM (n = 3). L-Name and Dexamethasone was used as positive control.

It was next investigated whether the inhibition of NO production was due to a decrease in iNOS expression or impairment of iNOS activity. iNOS is transcribed within 2-4 hours

and translated within six hours after LPS-stimulation in macrophages (Xie *et al.* 1994). As such, α-MG was added at a concentration close to IC_{50} value and NO was measured at 0, 6 or 14 h after RAW 264.7 macrophages stimulation with LPS. When α-MG was added simultaneously with the stimulus, about 50% of NO production was inhibited. When the compound was added 6 h after stimulation, the effect on iNOS was not detected (**Table 3**). This finds, support the hypothesis that the compound act in the first hours after LPS stimulation.

Once again, toxicity was excluded by MTT viability assay, since cells showed viability higher than 90% at dilution closest to IC_{50} of α-MG. Concentrations of xanthone and controls (L-NAME and Dexamethasone) used in this study was the dilution closest to IC_{50}.

Table 3: Inhibitory effect of α-MG on NO production by RAW264.7.

	NO inhibition (% of control)		
	0 h	6 h	14 h
α-MG at **12,5 µM**	44.7 ± 3.2 %	n.i.	n.i.
L-Name at **62,5 µM**	52.1 ± 6.0 %	50.7 ± 3.8 % $^{+}$	24.7 ± 3.1 % *
Dexamethasone at **6,25 µM**	55.9 ± 2.3 %	15.7 ± 6.0 % *	n.i.

Macrophages were exposed to LPS and treated with the alpha-mangostin at different times after stimulation: 0 h (simultaneously with the stimulus), 6 h and 14 h after stimulation. Results are the mean ± SEM (n = 3). n.i.= no inhibition. * $p < 0.001$, $^{+}$ $p > 0.05$. L-NAME and Dexamethasone was used as positive controls.

Effects of α-MG on macrophage production of IL-1β, IL-10, TGF-β1 and TNF-α

Further experiments were carried out to determine the effect of α-MG on the production of four cytokines by LPS-stimulated THP-1 macrophages cell line. Two concentrations of the compound (below GI_{50}) were studied in order to conduct a dose-response study and evaluate the effect of this xanthone on the kinetics of cytokine production.

A significant decrease in the production of IL-1β, TGF-β1 was observed for the two concentrations of α-MG tested in macrophages, as compared to α-MG-untreated macrophages, while an augment of TNF-α was detected. IL-10 expression was not affected (**Figure 10**).

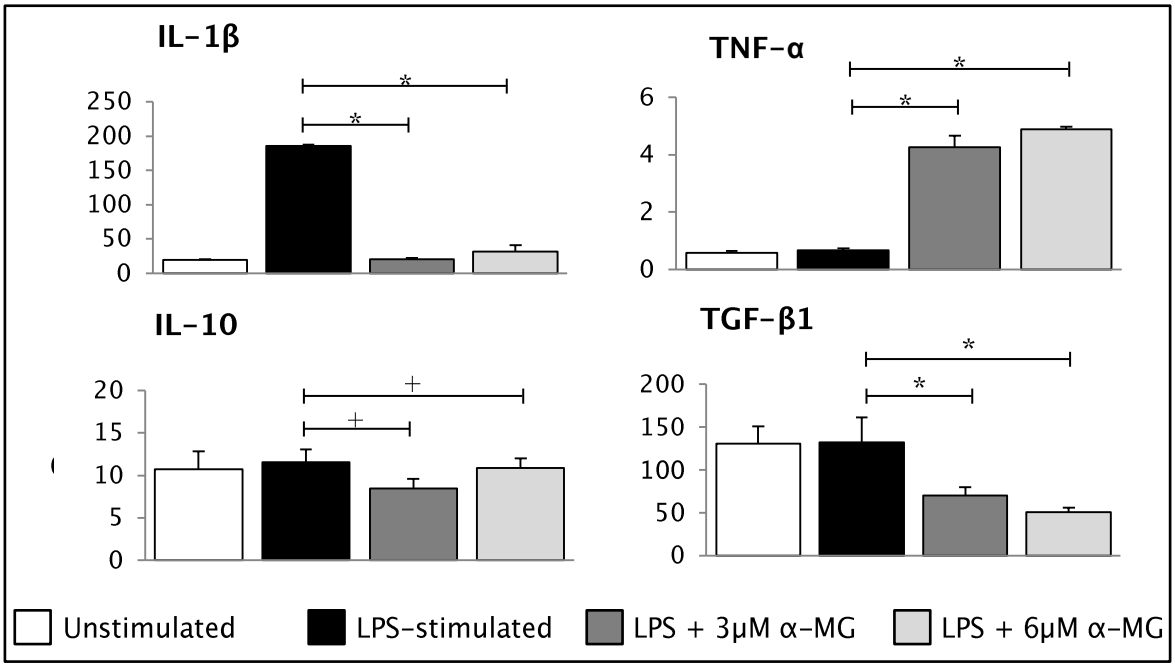


Figure 10: Production of IL-1β, IL-10, TGF-β1 and TNF-α by THP-1 macrophages. Cytokine production was evaluated on unstimulated macrophages (basal), LPS-stimulated macrophages and macrophages treated with 3 and 6 µM of alpha-mangostin. Data are the mean ± SEM from tree independent experiments performed in duplicate. * $p < 0.001$; $^{+}$ $p > 0.05$.

Effects on lymphocyte proliferation

The effect of α-MG on the mitogenic response of peripheral human lymphocytes to PHA, was evaluated (**Table 4**) and measured in concentration corresponding to 50 % inhibition of proliferation (IC_{50}). The compound strongly inhibited lymphocytes proliferation in a dose-dependent manner (data not show).

No lymphocytoxicity was observed when the human lymphocytes were exposed to the IC_{50} concentrations of these xanthones (cell viability near to 100%), which leads to the conclusion that its inhibitory activity was associated with cell proliferation rather than to a toxic effect (data not shown).

Table 4: Effect of α-MG on the PHA-induced proliferation on human lymphocytes

	Proliferation (IC_{50})
Alpha-mangostin	9.2 ± 0.3 µM
Cyclosporin A	0.1 ± 0.4 X 10^{-2} µM

Cyclosporin A was used as positive control. Results are the mean ± SEM of 3 independent experiments in duplicate.

Effects of α-MG on lymphocyte production of IL-1β, IL-10, TGF-β1 and TNF-α

The effect of α-MG at three concentration (below, above and IC_{50}) on cytokine production by PHA-simulated human mononuclear cells from peripheral blood was evaluated (**Figure 11**). At 20 µM (above IC_{50}) the compound showed a strong inhibition of IL-10 and TNF-α production and induction of TGF-β1 but no effects were detected at lower concentrations. The compound did not affect the production of IL-1β by PHA-stimulated lymphocytes compared with stimulated non-treated cells (positive control). No toxicity on lymphocytes was observed for the concentrations tested (viability > to 80%).

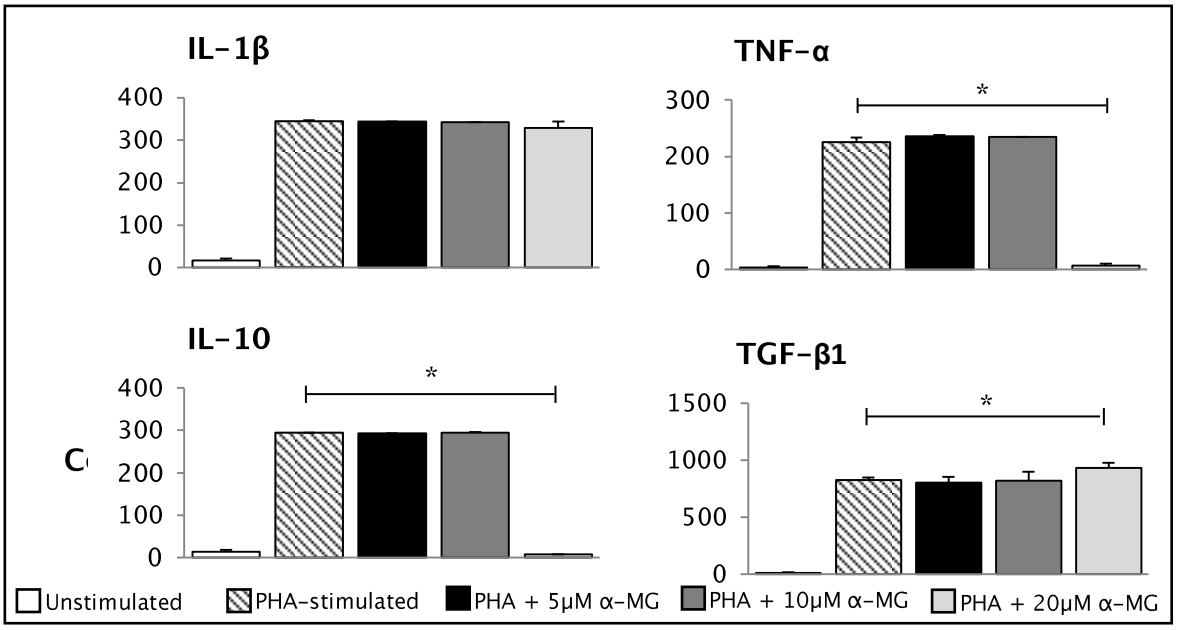


Figure 11: Production of IL-1β, IL-10, TGF-β1 and TNF-α by PHA-stimulated human mononuclear cells. Cytokines production was evaluated on unstimulated lymphocutes (basal), PHA-stimulated lymphocytes and lymphocytes treated with 5, 10 and 20 µM of α-mangostin. Data are the mean ± SEM from tree independent experiments, performed in duplicate. * $p < 0.001$.

Effect of α-MG on melanoma growth

The cell growth inhibitory effect of α-MG was examined against A375-C5 cell line. This xanthone is a potent inhibitor of the growth of A375-C5 (**Table 5**) in a dose-dependent manner (data not show).

Table 5: Effects of α-mangostin on the growth of A375-C5 human melanoma cell line.

	Growth inhibition (GI_{50})
Alpha-mangostin	11.3 ± 0.9 µM
Doxorrubicin	$1.8 \times 10^{-3} \pm 0.4 \times 10^{-3}$ µM

Results are the mean ± SEM (n = 3). Doxorrubicin was used as positive control.

Antitumor effect of conditioned macrophage culture medium

As modulation of the immune system has been a promising approach in melanoma treatment. It was investigated the possible influence of conditioned macrophage supernatants on A375-C5 cells growth to teste the influence of soluble immunologic factors produced by macrophages on melanoma cell line.

A significant ($p < 0.001$) decrease in melanoma cell growth was observed after exposure to supernatants from both α-MG-treated and untreated THP-1 macrophages (**Figure 12**), which is in favor of an α-MG-independent anti-tumor effect of soluble mediators produced by cultured THP-1 macrophages.

	Growth inhibition (% of control)
Alpha-mangostin (**6,25 µM**)	5.4 ± 2.9 %
Macrophages supernatants	25.0 ± 2.5 % *
α-MG (**6,25 µM**) conditioned macrophages supernatants	24.4 ± 2.7 % *

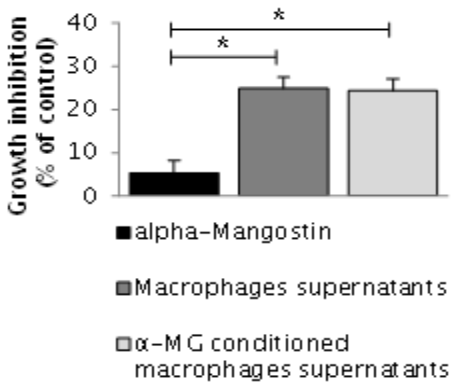


Figure 12: Antitumor effect of alpha-mangostin, macrophages supernatants and alpha-mangostin conditioned macrophage culture medium on A375-C5 melanoma cell line. THP-1 PMA-differentiated macrophages were treated with xanthone and supernatants were added to melanoma cells. Results show mean values ± SEM (n = 3). * $p < 0.001$

Discussion

Natural xanthones, including those from mangosteen extract have antitumor and immunomodulatory effects (Pinto *et al.* 2005; Obolskiy *et al.* 2009; Shan *et al.* 2011; Gutierrez-Orozco and Failla 2013). In particular, alpha-mangostin has been repeatedly associated with modulation of a great variety of physiologic pathways, including inflammation. These findings reinforce their antitumor potential, fighting tumor progression and treatment resistance (Pinto *et al.* 2005; Shan *et al.* 2011; Gutierrez-Orozco and Failla 2013).

In melanoma, several evidences reported the potential of immune targets as therapeutic agents. This facts result in the current use of ipilimumab, an immunomodulating antibody that target CTLA4 on activated T cells and inhibit regulatory T cells, in spite of the severe toxicity associated (Hodi *et al.* 2010; Graziani *et al.* 2012).

The character highly immunogenic of melanocytic tumors and the influence of host immune response and microenvironment inflammatory cells in cancer growth were

verified (Hussein 2004; Dranoff 2009; Chen *et al.* 2011). Indeed, primary melanomas undergo spontaneous regression much more frequently than any other cancer that may be a sign of immunosurveillance or, by other way, it may be due melanoma are easier to visualize (Printz 2001; Kalialis *et al.* 2009). Nevertheless, other evidences corroborate the involvement of immune system, namely the relatively amount of tumor-infiltrating lymphocytes in melanoma microenvironment compared with other cancers and associated with favorable prognostic (Maio 2012; Kushnir and Merimsky 2013) and the appearance of autoimmune condition as vitiligo in melanoma patients or patients treated with immunotherapy which normally means a better disease outcome (Le Gal *et al.* 2001; Phan *et al.* 2001; Boasberg *et al.* 2006). In more advanced stages, this cancer became capable to avoid immunosurveillance (Reiman *et al.* 2007; Speeckaert *et al.* 2011) and its progression and metastatic potential may be supported by immune cells present in tumor microenvironment, as the tumor-associated macrophages (TAMs) since they are the most abundant leucocytes in melanoma and represent a poor prognostic (Brocker *et al.* 1988; Bernengo *et al.* 2000; Makitie *et al.* 2001; Varney *et al.* 2005; Porta *et al.* 2007; Solinas *et al.* 2009; Mantovani and Sica 2010; Qian and Pollard 2010).

In spite of the verified interaction of immune system in melanoma treatment and the potential of xanthones as antitumor and immunomodulatory agent, any research was based on the direct effect of any immune cell treated with xanthonic compounds in the tumor growth.

According to the established influence of inflammation on cancer (induces carcinogenisis, tumor progression and promotes angiogenisis) (Apte and Voronov 2008; Germano *et al.* 2008; Kundu and Surh 2008; Colotta *et al.* 2009; Mantovani 2010) and particularly in melanoma, it was investigated the potential effect of the compound in different immune pathways in human and murine macrophage cell lines and human mononuclear cells from peripheral blood. Indeed, α-MG inhibited NO production and TNF-α secretion by murine macrophages cells (Chen *et al.* 2008; Tewtrakul *et al.* 2009). In spite of that finds, any studies were performed in the cytokines interleukin - 1β (IL-1β), IL-10, the tumor grow factor - β1 (TGF-β1) and the tumor necrosis factor - α (TNF- α) in THP-1 macrophages as performed in this paper.

RAW 264.7 was used for investigation of NO production, since this cell line was referred as an appropriate model to study iNOS system (Dirsch *et al.* 1998; Shih *et al.* 2010). Human macrophages *in vitro* produce low quantity of NO in response to stimuli (Thomassen and Kavuru 2001). However, either *in vitro* murine cells and *in vivo* human macrophages express great quantities of NO in inflammatory conditions (Ochoa *et al.* 1991; Dirsch *et al.* 1998)

α-MG was a strong inhibitor of NO production by LPS-stimulated RAW 264.7 (IC_{50} = 13.8 ± 0.7 μM). Since α-MG effect was not due to cell death or scavenging of chemicaly generated NO, it may act at the level of NO production by iNOS.

In inflammatory process, NO is produced by inducible NO synthetase (iNOS or NOS2) from L-arginine and molecular oxygen (Gross and Wolin 1995; Aramaki 2000). The inductible form is stimulated by imunologic factors and expressed in virtually all cells (Gross and Wolin 1995). In macrophages, its production is stimulated by a number of cytokines and LPS (Lowenstein *et al.* 1993; Gross and Wolin 1995). Production of NO could be a consequence of iNOS regulation at transcriptional and post-transcriptional level, depending of gene transcription, stability and translation of mRNA, stability of the protein, availability of substrates or co-factors, inhibitors that compete with substrates and the known NO feedback effect (Nathan and Xie 1994; Bogdan *et al.* 2000; Brunet 2001).

Therefore, the underlying mechanism to α-MG inhibition of NO production needed further investigation. For that, α-MG was added at different hours: 0h (simultaneously), 6h and 14h after RAW 264.7 macrophages stimulation with LPS.

In spite of α-MG exhibit a potent inhibition of NO production when added simultaneously with LPS-stimulation, this production remained stable either when it was added 6 or 14h after stimulation. This findings suggest an interference on iNOS expression since the enzyme is transcribed within 2-4 hours and translated within six hours after LPS-stimulation in macrophages (Xie *et al.* 1994). L-NAME (inhibitor of iNOS activity) (McCall *et al.* 1991), and Dexamethasone, inhibitor of iNOS expression (Korhonen *et al.* 2002) was used as positive controls and the obtained results was in accordance with the mechanism proposed to these inhibitors by our technique.

In accordance to our finds, Chen *et al* and Tewtrakul *et al* reported an inhibition of iNOS mRNA expression on RAW 264.7 macrophages by α-MG.

Inhibition of iNOS expression indicates an anti-inflammatory potential of α-MG. However, in cancer, this inhibition represents controversial effects because NO has been reported as cytotoxic in melanoma and many other tumors, but simultaneously it modulates many pro-tumor pathways including resistance to apoptosis, cell cycle progression, angiogenesis invasion, and metastasis (Ying and Hofseth 2007; Choudhari *et al.* 2013). So, the result of α-MG-dependent inhibition of NO production for melanoma progression needs further investigation.

Macrophages, including TAMs, are dynamic and heterogeneous cells mainly due to their capacity to respond to stimulus. According to the microenvironment they may be polarized into a spectrum of phenotypes between the extremes M1 (classic) and M2 (alternative). M1 macrophages produced high levels of iNOS, TNF-α, IL-1β and other pro-

inflammatory molecules, while M2 macrophages are immunosuppressive, significantly expressing IL-10 and TGF-β (Allavena *et al.* 2008; Biswas *et al.* 2013).

Several evidences indicate that macrophage phenotypes can change during tumor progression (Zaynagetdinov *et al.* 2011). M1 activation may induce chronic inflammation, a factor that could predispose to tumor initiation (Greten *et al.* 2004; Pikarsky *et al.* 2004). However, in early stages of tumor progression, TAMs adopt an M1-like phenotype that contributes to anti-tumor immunity. M2 phenotype is mainly expressed in established tumors and induce immunosuppressive, angiogenic and metastatic effects (Mantovani *et al.* 2004; Sica *et al.* 2006; Gordon and Mantovani 2011; Zaynagetdinov *et al.* 2011; Liao *et al.* 2014).

α-MG had a dual influence on the immune system, either inhibiting IL-1β and NO responses, or inhibiting TGF-β1 and stimulating TNF-α expression.

IL-1β, a pro-inflammatory cytokine mainly produced by monocytes and macrophages is an example of the pleiotropism of immune system. In melanoma its expression was associated to tumor progression and promotion of lung metastases from melanoma (Giavazzi *et al.* 1990; Meyer *et al.* 2011). Therefore, IL-1β has been associated with all steps of malignancy (carcinogenesis, progression, invasion and metastasis) and may even be expressed by the tumor cells (Apte and Voronov 2008). In contrast, M1 macrophages phenotype produced interleukin induce an immune response against malignant cells (Fairweather and Cihakova 2009). Considering all that was stated above, the inhibition of IL-1β mediated by α-MG could be beneficial so this cytokine induces tumor progression and metastasis. Melanoma is a highly metastatic kind of cancer contributing to the high mortality associated (Kuphal and Bosserhoff 2009; Raaijmakers *et al.* 2013), therefore a therapy that suppress or control angiogenesis and invasion may be an important approach.

TGF-β1 regulates multiple cellular processes, including proliferation and differentiation. It acts as a potent inducer of differentiation in normal cells which leads to the concept that this cytokine protects against cancer in early stages of carcinogenesis (Yang *et al.* 2008). By the other hand, several tumors express increasing amounts of TGF-β1 which is associated with disease progression, invasion and metastasis both *in vivo* and *in vitro* (Akhurst and Derynck 2001). In melanoma, TGF-β isoforms (TGF-β1/2/3) are highly expressed and increase in parallel with tumor progression (Krasagakis *et al.* 1998; Javelaud *et al.* 2008). According to that, this cytokine is commonly produced by M2-like macrophages leads to a Th2 response, i.e., promotes an immunosuppressive microenvironment that allows the tumor immune escape (Coffelt *et al.* 2009; Martinez *et al.* 2009; Siveen and Kuttan 2009; Hao *et al.* 2012; Sica and Mantovani 2012). The

inhibition of TGF-β1 by α-MG may benefit the treatment of melanoma, mainly in later stages of tumorigenesis.

TNF-α stimulation predicts a favorable outcome in melanoma treatment since several reports associated the use of TNF inhibitors as a factor that appear to increase the risk of skin cancer, including melanoma (Mariette *et al.* 2011; Kouklakis *et al.* 2013). In spite of that, the concentration expressed by THP-1 cells was minimal even in LPS-stimulated and non-treated macrophages.

IL-10 is an anti-inflammatory cytokine able to induce tumor progression through the promotion of angiogenesis and suppression of immune surveillance. In melanoma, its influence facilitates metastasis formation (García-Hernández *et al.* 2002; Itakura *et al.* 2011). In the present study, it was observed no alteration on IL-10 concentration after treatment with α-MG.

Taking into account the pattern of cytokines altered by the treatment with α-MG, it was inferred the potential underling mechanism of iNOS inhibition by xanthone. *NOS2* gene expression and translation was stimulated by a number of pro-inflammatory cytokines, particularly TNF-α, IL-1β and IFN-γ, compounds of bacterial origin as LPS (Nathan 1992) and stress signaling as hypoxia (Ferreiro *et al.* 2001). These stimuli may activate NF-κB or JAK-STAT (janus tyrosine kinase - signal transducers and activators of transcription) pathways (Kleinert *et al.* 1998; Xuan *et al.* 2001). Additionally, MAPK pathway most likely contributes to iNOS expression (Janssen-Heininger *et al.* 1999; Chan *et al.* 2001). It is also known that TGF-β1 inhibits iNOS expression by mRNA destabilization (Perrella *et al.* 1994). NF-κB pathway contributes to iNOS synthesis and upregulate IL-1β level. α-MG treated THP-1 macrophages expressed lower IL-1β and NO levels comparatively to control cells leading us to hypothesize the interference of the xanthone with NF-κB pathway. More studies must be performed in order to corroborate this supposition.

Macrophage activation and polarization are triggered by T helper cells (Th1 induce M1 phenotype and Th2 the M2-like phenotype). In turn, M1 macrophages recruit Th1 cells, CD8+ CTL (cytotoxic T cells) and NK cells leading to an inflammatory response and antitumor immunity, while M2-like macrophages favor the recruitment and development of Treg (regulatory T cells) and Th2, leading to a response that supports tumor growth through immunosuppression (Mantovani *et al.* 2002; Mantovani *et al.* 2004; Martinez *et al.* 2009; Siveen and Kuttan 2009; Sica and Mantovani 2012). In order to complement the described effect of α-MG on the immune microenvironment of melanoma, a study in influence of the compound in proliferation and cytokines production by human lymphocytes was performed. The compound strongly inhibit PHA-stimulated human mononuclear cells proliferation.

As weel as discussed to macrophages, lymphocytes have divergent roles in cancer due to their plasticity (Lakshmi Narendra *et al.* 2013). With the aim of establish the impact of proliferation inhibition in tumors, a set of four cytokines was evaluated in PHA-stimulated mononuclear cells from peripheral blood. At IC_{50} (≃ 10 μM) concentration of α-MG any cytokine concentration was altered. IL-10 and TNF-α was reduced to basal levels and TGF-β1 was stimulated at twice IC_{50} concentration. This finds indicate and absent effect of α-MG in T lymphocytes at concentrations required for inhibition of melanoma cells growth and for the immunomodulatory influence in macrophages which may indicate no significant impact of lymphocytes treated with α-MG on melanoma growth inhibition or in modulating of macrophages. Recently, Kasemwattanaroj *et al*, 2013 reported no alteration in IL-1β and TNF-α expression levels by conavalin A stimulated human mononuclear cells treated with α-MG (Kasemwattanaroj *et al.* 2013).

Considering the results of cytokines modulation in THP-1 human macrophages mediated by α-MG and the established involvement of immune system in melanoma treatment (Brocker *et al.* 1988; Bernengo *et al.* 2000; Makitie *et al.* 2001; Varney *et al.* 2005; Porta *et al.* 2007; Solinas *et al.* 2009; Mantovani and Sica 2010; Qian and Pollard 2010), it was hyphotisised if condicioned macrophages culture medium may potentiate the cytotoxic activity of α-MG. Anticancer effect of α-MG was previously evaluated in different melanoma cell lines (Wang *et al.* 2011; Wang *et al.* 2012a; Wang *et al.* 2012b; Wang *et al.* 2013; Beninati *et al.* 2014). In this work, the antitumor activity of α-MG on melanoma A375-C5 cells was determined and compared with that of α-MG treated macrophages supernatants. α-MG strongly inhibited melanoma growth (GI_{50} = 11.3 ± 0.9 μM). The results also demonstrated that the supernatants od THP-1 macrophages either treated or not with α-MG significantly interfere with melanoma cell growth. This results prove that the immune microenvironment created by macrophages associated to tumors interfere with tumor cell viability. For α-MG dose tested (6.25 μM), below GI_{50} concentration, no significantly differences were observed for the cytotoxic activity of macrophages supernatants, when treated or not with xanthone.

However, it was proved that α-MG interferes with cytokine and NO production by activated macrophages leading to the evidence that the xanthone interferes with immune microenvironment of the tumor. Therefore, it was expected a difference between the cytotoxic effect of treated and non-treated macrophages supernatants. Our results could be due to the lower dose used since for 6.25 μM only a ≃ 5% inhibition of melanoma cell growth was observed. Althought, no higher doses could be used in the assays since they were associated with a significant loss of viability of THP-1 macrophages, as determined by the MTT assay.

Therefore, further studies in other *in vitro* or/and *in vivo* models must be performed in order to clarify the α-MG effect in melanoma treatment and consequently establish α-MG as a prospective co-adjuvant drug in these tumors. From this set of data find in this study it is possible to infer that α-MG could be very interesting agent for melanoma treatment.

References

Akhurst RJ & Derynck R (2001) TGF-β signaling in cancer – a double-edged sword. Trends in Cell Biology 11: S44-S51.

Allavena P, Sica A, Solinas G *et al* (2008) The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. Crit Rev Oncol Hematol 66: 1-9.

Apte RN & Voronov E (2008) Is interleukin-1 a good or bad 'guy' in tumor immunobiology and immunotherapy? Immunological Reviews 222: 222-241.

Aramaki Y (2000) Liposomes as immunomodulator--inhibitory effect of liposomes on NO production from macrophages. Biol Pharm Bull 23: 1267-74.

Beninati S, Oliverio S, Cordella M *et al* (2014) Inhibition of cell proliferation, migration and invasion of B16-F10 melanoma cells by alpha-mangostin. Biochem Biophys Res Commun.

Bernengo MG, Quaglino P, Cappello N *et al* (2000) Macrophage-mediated immunostimulation modulates therapeutic efficacy of interleukin-2 based chemoimmunotherapy in advanced metastatic melanoma patients. Melanoma Res 10: 55-65.

Biswas SK, Allavena P & Mantovani A (2013) Tumor-associated macrophages: functional diversity, clinical significance, and open questions. Semin Immunopathol 35: 585-600.

Boasberg PD, Hoon DS, Piro LD *et al* (2006) Enhanced survival associated with vitiligo expression during maintenance biotherapy for metastatic melanoma. J Invest Dermatol 126: 2658-63.

Bogdan C, Rollinghoff M & Diefenbach A (2000) Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Curr Opin Immunol 12: 64-76.

Brocker EB, Zwadlo G, Holzmann B *et al* (1988) Inflammatory cell infiltrates in human melanoma at different stages of tumor progression. Int J Cancer 41: 562-7.

Brunet LR (2001) Nitric oxide in parasitic infections. Int Immunopharmacol 1: 1457-67.

Cerqueira F, Cidade H, Van Ufford L *et al* (2008) The natural prenylated flavone artelastin is an inhibitor of ROS and NO production. Int Immunopharmacol 8: 597-602.

Cerqueira F, Cordeiro-Da-Silva A, Araujo N *et al* (2003) Inhibition of lymphocyte proliferation by prenylated flavones: artelastin as a potent inhibitor. Life Sci 73: 2321-34.

Chan ED, Chan J & Schluger NW (2001) What is the Role of Nitric Oxide in Murine and Human Host Defense against Tuberculosis? American Journal of Respiratory Cell and Molecular Biology 25: 606-612.

Chanput W, Mes JJ & Wichers HJ (2014) THP-1 cell line: An in vitro cell model for immune modulation approach. Int Immunopharmacol.

Chanput W, Reitsma M, Kleinjans L *et al* (2012) beta-Glucans are involved in immune-modulation of THP-1 macrophages. Mol Nutr Food Res 56: 822-33.

Chen LG, Yang LL & Wang CC (2008) Anti-inflammatory activity of mangostins from Garcinia mangostana. Food Chem Toxicol 46: 688-93.

Chen P, Huang Y, Bong R *et al* (2011) Tumor-associated macrophages promote angiogenesis and melanoma growth via adrenomedullin in a paracrine and autocrine manner. Clinical cancer research : an official journal of the American Association for Cancer Research 17: 7230-9.

Choudhari SK, Chaudhary M, Bagde S *et al* (2013) Nitric oxide and cancer: a review. World J Surg Oncol 11: 118.

Coffelt SB, Hughes R & Lewis CE (2009) Tumor-associated macrophages: effectors of angiogenesis and tumor progression. Biochim Biophys Acta 1796: 11-8.

Colotta F, Allavena P, Sica A *et al* (2009) Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis 30: 1073-81.

Dirsch VM, Stuppner H & Vollmar AM (1998) The Griess assay: suitable for a bio-guided fractionation of anti-inflammatory plant extracts? Planta Med 64: 423-6.

Dranoff G (2009) Targets of protective tumor immunity. Ann N Y Acad Sci 1174: 74-80.

Fairweather D & Cihakova D (2009) Alternatively activated macrophages in infection and autoimmunity. J Autoimmun 33: 222-30.

Ferreiro CR, Chagas ACP, Carvalho MHC *et al* (2001) Influence of Hypoxia on Nitric Oxide Synthase Activity and Gene Expression in Children With Congenital Heart Disease: A Novel Pathophysiological Adaptive Mechanism. Circulation 103: 2272-2276.

García-Hernández ML, Hernández-Pando R, Gariglio P *et al* (2002) Interleukin-10 promotes B16-melanoma growth by inhibition of macrophage functions and induction of tumour and vascular cell proliferation. Immunology 105: 231-243.

Gast A, Bermejo JL, Claus R *et al* (2011) Association of inherited variation in Toll-like receptor genes with malignant melanoma susceptibility and survival. PLoS One 6: e24370.

Germano G, Allavena P & Mantovani A (2008) Cytokines as a key component of cancer-related inflammation. Cytokine 43: 374-379.

Giavazzi R, Garofalo A, Bani MR *et al* (1990) Interleukin 1-induced augmentation of experimental metastases from a human melanoma in nude mice. Cancer Res 50: 4771-5.

Gordon S & Mantovani A (2011) Diversity and plasticity of mononuclear phagocytes. Eur J Immunol 41: 2470-2.

Graziani G, Tentori L & Navarra P (2012) Ipilimumab: A novel immunostimulatory monoclonal antibody for the treatment of cancer. Pharmacological Research 65: 9-22.

Greten FR, Eckmann L, Greten TF *et al* (2004) IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell 118: 285-96.

Gross SS & Wolin MS (1995) Nitric oxide: pathophysiological mechanisms. Annu Rev Physiol 57: 737-69.

Gupta S, Rodrigues LM, Esteves AP *et al* (2008) Synthesis of N-aryl-5-amino-4-cyanopyrazole derivatives as potent xanthine oxidase inhibitors. Eur J Med Chem 43: 771-80.

Gutierrez-Orozco F & Failla ML (2013) Biological activities and bioavailability of mangosteen xanthones: a critical review of the current evidence. Nutrients 5: 3163-83.

Hao N-B, Lü M-H, Fan Y-H *et al* (2012) Macrophages in Tumor Microenvironments and the Progression of Tumors. Clinical and Developmental Immunology 2012: 11.

He X, Shu J, Xu L *et al* (2012) Inhibitory effect of Astragalus polysaccharides on lipopolysaccharide-induced TNF-a and IL-1beta production in THP-1 cells. Molecules 17: 3155-64.

Hodi FS, O'day SJ, Mcdermott DF *et al* (2010) Improved Survival with Ipilimumab in Patients with Metastatic Melanoma. New England Journal of Medicine 363: 711-723.

Hussein MR (2004) Genetic pathways to melanoma tumorigenesis. J Clin Pathol 57: 797-801.

Itakura E, Huang RR, Wen DR *et al* (2011) IL-10 expression by primary tumor cells correlates with melanoma progression from radial to vertical growth phase and development of metastatic competence. Mod Pathol 24: 801-9.

Janssen-Heininger YMW, Macara I & Mossman BT (1999) Cooperativity between Oxidants and Tumor Necrosis Factor in the Activation of Nuclear Factor (NF)- κ B. American Journal of Respiratory Cell and Molecular Biology 20: 942-952.

Javelaud D, Alexaki VI & Mauviel A (2008) Transforming growth factor-beta in cutaneous melanoma. Pigment Cell Melanoma Res 21: 123-32.

Kalialis LV, Drzewiecki KT & Klyver H (2009) Spontaneous regression of metastases from melanoma: review of the literature. Melanoma Res 19: 275-82.

Kasemwattanaroj P, Moongkarndi P, Pattanapanyasat K *et al* (2013) Immunomodulatory activities of alpha-mangostin on peripheral blood mononuclear cells. Nat Prod Commun 8: 1257-60.

Kleinert H, Wallerath T, Fritz G *et al* (1998) Cytokine induction of NO synthase II in human DLD-1 cells: roles of the JAK-STAT, AP-1 and NF-κB-signaling pathways. British Journal of Pharmacology 125: 193-201.

Kono K, Salazar-Onfray F, Petersson M *et al* (1996) Hydrogen peroxide secreted by tumor-derived macrophages down-modulates signal-transducing zeta molecules and inhibits tumor-specific T cell-and natural killer cell-mediated cytotoxicity. Eur J Immunol 26: 1308-13.

Korhonen R, Lahti A, Hamalainen M *et al* (2002) Dexamethasone inhibits inducible nitric-oxide synthase expression and nitric oxide production by destabilizing mRNA in lipopolysaccharide-treated macrophages. Mol Pharmacol 62: 698-704.

Kouklakis G, Efremidou EI, Pitiakoudis M *et al* (2013) Development of primary malignant melanoma during treatment with a TNF-alpha antagonist for severe Crohn's disease: a case report and review of the hypothetical association between TNF-alpha blockers and cancer. Drug Des Devel Ther 7: 195-9.

Krasagakis K, Tholke D, Farthmann B *et al* (1998) Elevated plasma levels of transforming growth factor (TGF)-beta1 and TGF-beta2 in patients with disseminated malignant melanoma. Br J Cancer 77: 1492-4.

Kundu JK & Surh YJ (2008) Cancer chemopreventive and therapeutic potential of resveratrol: mechanistic perspectives. Cancer Lett 269: 243-61.

Kuphal S & Bosserhoff A (2009) Recent progress in understanding the pathology of malignant melanoma. J Pathol 219: 400-9.

Kushnir I & Merimsky O (2013) The evolution in melanoma treatment as a reflection of precision-oriented medicine. Oncol Lett 5: 424-426.

Lakshmi Narendra B, Eshvendar Reddy K, Shantikumar S *et al* (2013) Immune system: a double-edged sword in cancer. Inflamm Res 62: 823-34.

Le Gal FA, Avril MF, Bosq J *et al* (2001) Direct evidence to support the role of antigen-specific CD8(+) T cells in melanoma-associated vitiligo. J Invest Dermatol 117: 1464-70.

Liao Q, Zeng Z, Guo X *et al* (2014) LPLUNC1 suppresses IL-6-induced nasopharyngeal carcinoma cell proliferation via inhibiting the Stat3 activation. Oncogene 33: 2098-109.

Lou X, Hou Y & Liang D (2013) Effects of hepatitis B virus X protein on human T cell cytokines. Can J Microbiol 59: 620-6.

Lowenstein CJ, Alley EW, Raval P *et al* (1993) Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. Proc Natl Acad Sci U S A 90: 9730-4.

Maio M (2012) Melanoma as a model tumour for immuno-oncology. Ann Oncol 23 Suppl 8: viii10-4.

Makitie T, Summanen P, Tarkkanen A *et al* (2001) Tumor-infiltrating macrophages (CD68(+) cells) and prognosis in malignant uveal melanoma. Invest Ophthalmol Vis Sci 42: 1414-21.

Mantovani A (2010) Molecular pathways linking inflammation and cancer. Curr Mol Med 10: 369-73.

Mantovani A, Allavena P & Sica A (2004) Tumour-associated macrophages as a prototypic type II polarised phagocyte population: role in tumour progression. Eur J Cancer 40: 1660-7.

Mantovani A & Sica A (2010) Macrophages, innate immunity and cancer: balance, tolerance, and diversity. Curr Opin Immunol 22: 231-7.

Mantovani A, Sozzani S, Locati M *et al* (2002) Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 23: 549-55.

Mariette X, Matucci-Cerinic M, Pavelka K *et al* (2011) Malignancies associated with tumour necrosis factor inhibitors in registries and prospective observational studies: a systematic review and meta-analysis. Ann Rheum Dis 70: 1895-904.

Martinez FO, Helming L & Gordon S (2009) Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol 27: 451-83.

Mccall TB, Feelisch M, Palmer RMJ *et al* (1991) Identification of N-iminoethyl-l-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells. British Journal of Pharmacology 102: 234-238.

Meyer C, Sevko A, Ramacher M *et al* (2011) Chronic inflammation promotes myeloid-derived suppressor cell activation blocking antitumor immunity in transgenic mouse melanoma model. Proc Natl Acad Sci U S A 108: 17111-6.

Monks A, Scudiero D, Skehan P *et al* (1991) Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. J Natl Cancer Inst 83: 757-66.

Nathan C (1992) Nitric oxide as a secretory product of mammalian cells. Faseb j 6: 3051-64.

Nathan C & Xie QW (1994) Regulation of biosynthesis of nitric oxide. J Biol Chem 269: 13725-8.

Obolskiy D, Pischel I, Siriwatanametanon N *et al* (2009) Garcinia mangostana L.: a phytochemical and pharmacological review. Phytother Res 23: 1047-65.

Ochoa JB, Udekwu AO, Billiar TR *et al* (1991) Nitrogen oxide levels in patients after trauma and during sepsis. Ann Surg 214: 621-6.

Pedro M, Cerqueira F, Sousa ME *et al* (2002) Xanthones as inhibitors of growth of human cancer cell lines and their effects on the proliferation of human lymphocytes in vitro. Bioorganic & medicinal chemistry 10: 3725-30.

Perrella MA, Yoshizumi M, Fen Z *et al* (1994) Transforming growth factor-beta 1, but not dexamethasone, down-regulates nitric-oxide synthase mRNA after its induction by interleukin-1 beta in rat smooth muscle cells. J Biol Chem 269: 14595-600.

Phan GQ, Attia P, Steinberg SM *et al* (2001) Factors associated with response to high-dose interleukin-2 in patients with metastatic melanoma. J Clin Oncol 19: 3477-82.

Pikarsky E, Porat RM, Stein I *et al* (2004) NF-kappaB functions as a tumour promoter in inflammation-associated cancer. Nature 431: 461-6.

Pinto MM, Sousa ME & Nascimento MS (2005) Xanthone derivatives: new insights in biological activities. Curr Med Chem 12: 2517-38.

Porta C, Subhra Kumar B, Larghi P *et al* (2007) Tumor promotion by tumor-associated macrophages. Adv Exp Med Biol 604: 67-86.

Printz C (2001) Spontaneous regression of melanoma may offer insight into cancer immunology. J Natl Cancer Inst 93: 1047-8.

Qian BZ & Pollard JW (2010) Macrophage diversity enhances tumor progression and metastasis. Cell 141: 39-51.

Raaijmakers MI, Rozati S, Goldinger SM *et al* (2013) Melanoma immunotherapy: historical precedents, recent successes and future prospects. Immunotherapy 5: 169-82.

Reiman JM, Kmieciak M, Manjili MH *et al* (2007) Tumor immunoediting and immunosculpting pathways to cancer progression. Semin Cancer Biol 17: 275-87.

Roreno (2013) Registo Oncológico Nacional 2007.

Shan T, Ma Q, Guo K *et al* (2011) Xanthones from mangosteen extracts as natural chemopreventive agents: potential anticancer drugs. Curr Mol Med 11: 666-77.

Shih MF, Cheng YD, Shen CR *et al* (2010) A molecular pharmacology study into the anti-inflammatory actions of Euphorbia hirta L. on the LPS-induced RAW 264.7 cells through selective iNOS protein inhibition. J Nat Med 64: 330-5.

Sica A & Mantovani A (2012) Macrophage plasticity and polarization: in vivo veritas. J Clin Invest 122: 787-95.

Sica A, Schioppa T, Mantovani A *et al* (2006) Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. Eur J Cancer 42: 717-27.

Siveen KS & Kuttan G (2009) Role of macrophages in tumour progression. Immunol Lett 123: 97-102.

Smalley KS, Haass NK, Brafford PA *et al* (2006) Multiple signaling pathways must be targeted to overcome drug resistance in cell lines derived from melanoma metastases. Mol Cancer Ther 5: 1136-44.

Solinas G, Germano G, Mantovani A *et al* (2009) Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. J Leukoc Biol 86: 1065-73.

Solit DB & Rosen N (2011) Resistance to BRAF Inhibition in Melanomas. New England Journal of Medicine 364: 772-774.

Speeckaert R, Van Geel N, Vermaelen KV *et al* (2011) Immune reactions in benign and malignant melanocytic lesions: lessons for immunotherapy. Pigment Cell Melanoma Res 24: 334-44.

Teixeira M, Cerqueira F, Barbosa CM *et al* (2005) Improvement of the inhibitory effect of xanthones on NO production by encapsulation in PLGA nanocapsules. J Drug Target 13: 129-35.

Tewtrakul S, Wattanapiromsakul C & Mahabusarakam W (2009) Effects of compounds from Garcinia mangostana on inflammatory mediators in RAW264.7 macrophage cells. J Ethnopharmacol 121: 379-82.

Thomassen MJ & Kavuru MS (2001) Human alveolar macrophages and monocytes as a source and target for nitric oxide. Int Immunopharmacol 1: 1479-90.

Varney ML, Johansson SL & Singh RK (2005) Tumour-associated macrophage infiltration, neovascularization and aggressiveness in malignant melanoma: role of monocyte chemotactic protein-1 and vascular endothelial growth factor-A. Melanoma Res 15: 417-25.

Wang JJ, Sanderson BJ & Zhang W (2012a) Significant anti-invasive activities of alpha-mangostin from the mangosteen pericarp on two human skin cancer cell lines. Anticancer Res 32: 3805-16.

Wang JJ, Sanderson BJS & Zhang W (2011) Cytotoxic effect of xanthones from pericarp of the tropical fruit mangosteen (Garcinia mangostana Linn.) on human melanoma cells. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 49: 2385-91.

Wang JJ, Shi QH, Zhang W *et al* (2012b) Anti-skin cancer properties of phenolic-rich extract from the pericarp of mangosteen (Garcinia mangostana Linn.). Food and Chemical Toxicology 50: 3004-3013.

Wang JJ, Zhang W & Sanderson BJ (2013) Altered mRNA expression related to the apoptotic effect of three xanthones on human melanoma SK-MEL-28 cell line. Biomed Res Int 2013: 715603.

Wang T, Ge Y & Xiao M (2012c) Melanoma-derived conditioned media efficiently induce the differentiation of monocytes to macrophages that display a highly invasive gene signature. Pigment Cell & … 25: 493-505.

Xie QW, Kashiwabara Y & Nathan C (1994) Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. J Biol Chem 269: 4705-8.

Xuan Y-T, Guo Y, Han H *et al* (2001) An essential role of the JAK-STAT pathway in ischemic preconditioning. Proceedings of the National Academy of Sciences 98: 9050-9055.

Yang G, Li Y, Nishimura EK *et al* (2008) Inhibition of PAX3 by TGF-beta modulates melanocyte viability. Mol Cell 32: 554-63.

Ying L & Hofseth LJ (2007) An emerging role for endothelial nitric oxide synthase in chronic inflammation and cancer. Cancer Res 67: 1407-10.

Zaynagetdinov R, Sherrill TP, Polosukhin VV *et al* (2011) A critical role for macrophages in promotion of urethane-induced lung carcinogenesis. J Immunol 187: 5703-11.

VI. Synthetic xanthone: 1,2-dihydroxyxanthone

Paper 3

Paper 3 (draft): "1,2-dihydroxyxanthone antitumor activity: cytotoxicity and influence on the immune system microenvironment"

Abstract

Xanthones has been suggested as prospective candidates for cancer treatment. In melanoma its potential anticancer activity is, not only, due to a direct cytotoxic effect, but also, due to immunomodulation, since melanoma is highly immunogenic.

The main gold of this study was evaluate the 1,2-dihydroxyxanthone (i) cytotoxic effect on A375-C5 melanoma cell line and (ii) it interference with the immune macrophages induced microenvironment of the tumor.

1,2-Dihydroxyxanthone showed a moderate inhibition of melanoma growth (GI_{50} = 55.0 ± 2.3 µM). However, it strongly interferes with THP-1 human macrophages immune microenvironment, resulting in a two-fold increase of the cytotoxic effect of macrophages on A375-C5 melanoma cells. 1,2-Dihydroxyxanthone also decreased the concentration of IL-1β and stimulated TNF-α (characteristic cytokines of M1 phenotype) and inhibited IL-10 and increased TGF-β1 expression (characteristic cytokines of M2 phenotype). This xanthone also inhibited nitric oxide production (IC_{50} = 22.0 ± 0,9 µM) by RAW 264.7 murine macrophages, possibly by inhibit the synthesis of inducible nitric oxide synthase.

In conclusion, these finds allowed to infer a prospective impact of 1,2-dihydroxyxanthone in melanoma treatment, not only due to cytotoxic effect but also due to immunomodulation of tumor microenvironment.

Introduction

Melanoma is originated from melanocytic cells and is a result a multistep tumorigenesis. In past years the involvement of immune system in this process has been pointed as the main factor for progression of malignancy (Hussein 2004; Chen *et al.* 2011). According to that, many investigations have been focused on therapies with immune targets which result in the approved therapy Ipilimumab (inhibitor of the immunosuppressive regulatory T cells) (Dranoff 2009; Raaijmakers *et al.* 2013). However, the actual therapies, standard or alternatives are only effectives for a brief period of time due to escape mechanisms, and/or are associated with severe side effects (Hodi *et al.* 2010; Solit and Rosen 2011; Graziani *et al.* 2012).

Nowadays, there is a need to find more efficient and safe alternative treatments that target multiple pathways to prevent refractory tumor and consequently avoid the massive mortality rate associated to melanoma (Kuphal and Bosserhoff 2009; Gast *et al.* 2011; Raaijmakers *et al.* 2013).

The investigation in xanthonic compounds has been rising mostly due to their remarkable characteristics in the improvement of human health (Pinto *et al.* 2005; Pinto and Castanheiro 2009). Xanthones from natural origin are, in fact, very promising compounds, however, are limited in position or type of constituents due to the biosynthetic pathways. Synthesis of new xanthones is important to enlarge the diversity of compounds and increase the possibility of biological activities, creating compounds with better therapeutic proprieties (Pedro *et al.* 2002).

Our group has a vast experience in the syntheses of xanthonic derivatives. 1,2-dihidroxyxanthone (1,2-DHX) (**Figure 13**) was obtained by demethylation of 1,2-dimethoxyxanthone according to the described procedure (48%) (Gottlieb *et al.* 1970; Vermes *et al.* 1985). The antitumor activity of this compound was already evaluated in three cancer cell lines, namely UACC-62 (melanoma), MCF-7 (breast) and TK-10 (renal), showing specificity for melanoma cells (Pedro *et al.* 2002; Sousa *et al.* 2002). Studies of this compound were also carrying out in T lymphocytes and demonstrated a weak, but present, inhibition of their proliferation (Pedro *et al.* 2002; Sousa *et al.* 2002).

As immune system, mainly the tumor-associated macrophages (TAMs), are important in melanoma therapies, not only because the highly immunogenic character of melanocytic tumors, but also because macrophages are the most abundant immune cell present in the tumor (Brocker *et al.* 1988; Bernengo *et al.* 2000; Makitie *et al.* 2001; Varney *et al.* 2005; Porta *et al.* 2007; Solinas *et al.* 2009; Mantovani and Sica 2010; Qian and Pollard 2010). The cytotoxic activity of 1,2-DHX against A375-C5 melanoma epithelial cell line and its interference in immunomodulation of THP-1 macrophages was evaluated.

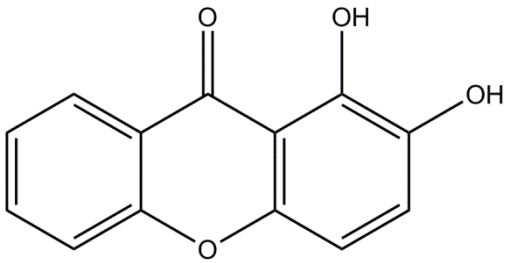


Figure 13: 1,2-Dihydroxyxanthone

Material and Methods

Chemicals and reagents

Reagents used in cell culture, including RPMI-1640, DMEM medium, and fetal bovine serum (FBS) were purchased from Gibco® Invitrogen Co. (Barcelona, Spain), 2-mercaptoethanol for synthesis was obtained from Merck (Whitehouse Station, NJ, USA), Glutamine cell culture grade and Dimethyl sulfoxide (DMSO) from Applichem (Darmstadt, Germany). N.N-Dimetilformamida (DMF) was acquiring from Spectramol Science Incorporated.

Reagents used in SRB assay as Trichloroacetic acid (TCA), Acetic acid glacial 99-100% and Trizma base were purchased from Prolabo (Oeiras, Portugal), CHEM-LAB (Zedelgem, Belgium) and Frilabo (Maia, Portugal), respectively.

IL-1β, IL-10, TNF-α and TGF-β1 ELISA Ready-Set-Go Kits were acquiered from eBioscience (San Diego, CA, USA).

All other chemicals of analytical grade used in the experiments and unless otherwise indicated were purchased from Sigma-Aldrich® (St. Louis, MO, USA).

Xanthone

1,2-Dihydroxyxanthone (1,2-DHX) was synthetized in Laboratory of Organic and Pharmaceutic Chemistry (Faculty of Pharmacy, University of Porto) and in CEQUIMED (Centro de Química Medicinal; University of Porto) as previously described (Gales *et al.* 2001) and stock solution kept at -20ºC in DMSO. Just prior each assay, stock solution was diluted in appropriate complete medium to the maximum concentration to test and 1:2 dilutions were serially prepared.

Cell lines

A375-C5 human malignant melanoma cell line and RAW 264.7 mouse macrophage cell line are part of CEQUIMED cell culture collection. THP-1 human monocyte cell line was a courtesy of Rui Appelberg.

A375-C5 was routinely maintained in 25 cm^2 flasks containing RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1 µL mL^{-1} Gentamycin at 37ºC in a humidified incubator with 5% CO_2. For the THP-1 cell line the culture medium just described was also supplemented with 0.05 mM 2-mercaptoethanol. RAW 264.7 cell line was maintained 75 cm^2 flasks containing DMEM medium with the 10% FBS and gentamycin, in the same conditions.

Cell lines were sub-cultured every 2 or 3 days by trypsinization (A375-C5), replacement of the medium (THP-1) or scraping (RAW 264.7) and used for each experiment when the cells were in exponential growth.

Human mononuclear cells were isolated from heparinized peripheral blood of healthy volunteers by Histopaque-1077 density centrifugation according to manufactures' instruction. Informed consent was obtained from each volunteer. Cells were ressuspended in RPMI medium supplemented with 10% FBS and 1 µL mL^{-1} gentamycin.

Sulphorrodamine B (SRB) growth inhibition assay

1,2-DHX effect on the growth of A375-C5 human melanoma cell line (7,5 X 10^4 cells mL^{-1}) was evaluated according to the method adopted by the National Cancer Institute (NCI, USA) (Monks *et al.* 1991) as already described by our group (Pedro *et al.* 2002; Gupta *et al.* 2008). Doxorubicin (1:10 dilutions) was used as positive control (Pedro *et al.* 2002; Gupta *et al.* 2008).

Antitumor effect of conditioned macrophages culture medium

After testing the cytotoxic effect of 1,2-DHX on A375-C5 melanoma cell line, the antitumor effect of conditioned macrophage culture medium was evaluated according to He *et al*, 2012. Briefly, THP-1 cell line was plated at 1 X 10^5 cells mL^{-1} and differentiated into THP-1 macrophages with 10 ng mL^{-1} PMA for 72h (He *et al.* 2012). Once differentiated, cells were washed twice with complete medium and left for another 24h incubation in order to obtain the resting stages of macrophages (Chanput *et al.* 2012; Chanput *et al.* 2014). Then, they were stimulated with 100 µL of LPS solution (1 µg mL^{-1}) and treated with 100 µL 1,2-DHX (concentration below GI_{50}). Treated cell were incubated for 24h at 37ºC, 5% CO_2 in a humidified incubator (He *et al.* 2012). Plates were centrifuged and half of the volume of each well was transferred to A375-C5 adherent cell monolayers, previously plated as for cancer growth inhibition assay procedure. After 48h incubation, SRB assay was performed, absorbance was measured and cell-growth inhibition determinate (Pedro *et al.* 2002).

Cytokine quantification

Culture supernatants of differentiated THP-1 cells, stimulated and treated as previously described, were storage at -20ºC until cytokine analyses.

Levels of interleukin-1β (IL-1β), interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGFβ1) in culture supernatants were quantified by ELISA Ready-Set-Go Kits (eBioscience) according to manufacturers' instructions and

measured at 510 nm in an ELISA reader (Stat FAX3200, Awareness Technology) (Cerqueira *et al.* 2003).

NO production assay

The determination of nitric oxide (NO) production by RAW 264.7 (1 X 10^6 cells mL^{-1}) after treatment and stimulation with 1.5 µg mL^{-1} LPS was quantified by Griess assay as previous described by our group (Teixeira *et al.* 2005; Cerqueira *et al.* 2008). *N*-nitro-L-arginine methyl ester (L-NAME), and inhibitor of inductive nitric oxide synthase (iNOS) activity, and Dexamethasone, an inhibitor of iNOS expression, were used as positive controls.

Since inhibition of NO production could be a consequence of an inhibition of iNOS expression and/or activity, it was necessary to investigate 1,2-DHX mechanism. For that, the compound was also added 6h and 14h after RAW 264.7 macrophages stimulation with LPS (Cerqueira *et al.* 2008).

NO Scavenging assay

To discard NO scavenging effect by xanthone, nitrite was chemically generated using sodium nitroprusside as previously described. Nitrite was quantified by Griess assay (Teixeira *et al.* 2005; Cerqueira *et al.* 2008).

MTT-viability assay

Toxicity of 1,2-DHX on THP-1 cell line and RAW 254.7 cell line was evaluated by MTT-viability assay (Pedro *et al.* 2002; Cerqueira *et al.* 2003; Teixeira *et al.* 2005).

Statistical analysis

Except otherwise stated, results are the mean ± SEM of at least three independent experiments, performed in duplicate. Statistical analysis was performed with SPSS for Windows (version 20.0). Statistical significance between groups was calculated by Mann-Whitney Test and it is considered for p values less than 0.05.

Ethics

Ethics approval was obtained by the Ethic Comity of University Fernando Pessoa.

Results

Antitumor effect of 1,2-DHX and conditioned macrophages culture medium

The evaluation of 1,2-DHX effect on A375-C5 melanoma growth was performed by SRB assay and revealed a moderate inhibitory effect of the xanthone (**Table 6**). This effect showed to be dose-dependent (data not show).

Table 6: Effect of 1,2-dihydroxyxanthone on the growth of A375-C5 human melanoma cell line.

	Growth inhibition (GI_{50})
1,2-DHX	55.0 ± 2.3 µM
Doxorrubicin	$1.8 \times 10^{-3} \pm 0.4 \times 10^{-3}$ µM

Results are the mean ± SEM of three independent experiments performed in duplicate. Doxorrubicin was used as positive control.

As modulation of immune system has been a promising approach in melanoma treatment, it was investigate the possible influence of 1,2-DHX conditioned macrophages supernatants on A375-C5 cells growth.

The results demonstrated a similar inhibition of cancer cells growth by the compound at 50 µM and non-treated macrophages. However, xanthone-treated macrophages-conditioned medium showed a significant ($p < 0,001$) decrease of melanoma cell growth comparatively with the other two conditions, as demonstrated in

Figure 14.

	Grow inhibition (% of control)
1,2-DHX (**50 µM**)	31.6 ± 4.9 %
Macrophages supernatants	25.0 ± 2.5 %
1,2-DHX (**50 µM**) conditioned macrophages supernatants	55.4 ± 4.4 % *

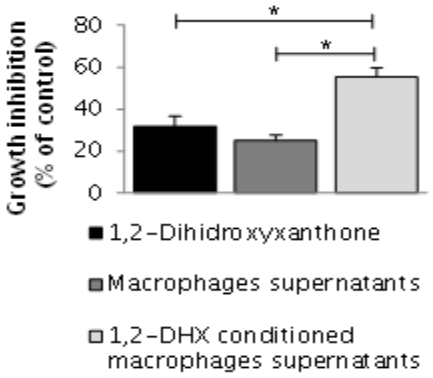


Figure 14: Cytotoxic activity of 1,2-Dihydroyixanthone, macrophages supernatants and 1,2-dihydroxyxanthone conditioned macrophages supernatants on A365-C5 melanoma cell line.THP-1 PHA-differentiated macrophages were treated with the compound and supernatants were added to melanoma cells. Results show mean values ± SEM (n = 3). * $p < 0.001$

Effect on IL-1β, IL-10, TGF-β1 and TNF-α production by macrophages

Further experiments were carried out to determine the underling mechanism of 1,2-DHX on macrophages. Two concentrations of the compound (over and below GI_{50}) were studied in order to evaluate the effect of this xanthone on the kinetics of cytokines production by LPS-stimulated THP-1 macrophages cell line (**Figure 15**). The expression of four cytokines was evaluated: IL-1β and TNF-α characteristic of M1 phenotype and IL-10 and TGF-β1 characteristic of M2 phenotype.

1,2-DHX significantly inhibited the expression of IL-1β and stimulated the expression of TNF-α at the two concentrations. At concentration above GI_{50}, xanthone stimulated expression of TGF-β1 and suppressed IL-10 by THP-1 macrophages.

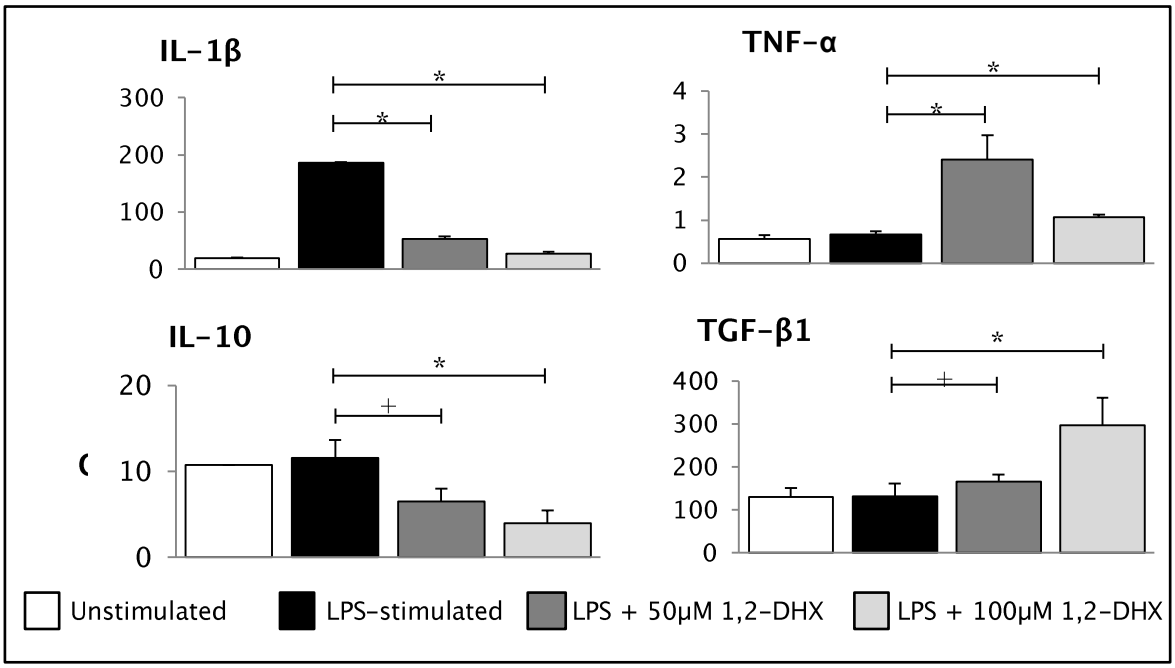


Figure 15: Effect of 1,2-DHX on IL-1β, IL-10, TGF-β1 and TNF-α production by THP-1 macrophages. Cytokines production was evaluated on Unstimulates macrophages (basal), LPS-stimulated macrophages (positive control) and macrophages treated with 6 and 3 µM 1,2-dihydroxyxanthone. Data are the mean ± SEM from one experiment, performed with duplicate cultures, and it representative of tree experiments carried out independently. * $p < 0.001$; $^{+}$ $p > 0.05$

Effect on NO production by macrophages

The production of NO by RAW 264.7 murine macrophages cell line was strongly inhibited by 1,2-DHX (IC_{50} = 22.0 ± 0.9 µM) (**Table 7**) in a dose-dependent manner (data not show).

NO inhibiton was not associated to cell death since at IC_{50} values, viability was near 100% (**Table 7**). 1,2-DHX did not show any scavenging activity of NO generated in a cell-free system (data not show).

Table 7: Effect of 1,2-DHX on NO production by LPS-stimulated RAW 264.7 macrophages

	NO inhibition (IC_{50})	Viability at IC_{50}
1,2-Dihydroxyxanthone	22.0 ± 0.9 µM	100 %
L-Name	62.4 ± 7.8 µM	96.0 ± 2.5 %
Dexamethasone	4.3 ± 0.6 µM	100 %

Results are the mean ± SEM (n = 3). L-Name and Dexamethasone was used as positive control.

It was next investigated whether the inhibition of NO production was due to a decrease in iNOS expression or impairment of iNOS activity. iNOS is transcribed within 2-4 hours and translated within six hours after LPS-stimulation in macrophages (Xie *et al.* 1994). As such, 1,2-DHX was added at a concentration close to IC50 value and NO was measured at 0, 6 and 14 h after RAW 264.7 macrophages stimulation with LPS. When 1,2-DHX was added simultaneously with the stimulus, about 57% of NO production was inhibited. When the compound was added 6 h after stimulation, the effect significantly decreased to 26% ($p < 0.001$), while a lack of inhibitory effect was observed when the compound was added 14 h after stimulation (**Table 8**).

Once again, toxicity was excluded by MTT viability assay, since cells showed viability higher than 90 % at dilution closest to IC_{50} of 1,2-DHX. Concentrations of xanthone and controls (L-NAME and Dexamethasone) used in this study were the dilution closest to IC_{50}, previously determined.

Table 8: Inhibitory effect of 1,2-DHX on NO production by RAW264.7.

	NO inhibition (% of control)		
	0 h	6 h	14 h
1,2-DHX at **25 µM**	56.6 ± 1.8 %	25.0 ± 2.8 % *	n.i.
L-Name at **62,5 µM**	52.1 ± 6.0 %	50.7 ± 3.8 % †	24.7 ± 3.1 % *
Dexamethasone at **6,25 µM**	55.9 ± 2.3 %	15.7 ± 6.0 % *	n.i.

Macrophages were exposed to LPS and treated with 1,2-dihydroxyxanthone at different times after stimulation: 0 h (simultaneously with the stimulus), 6 h and 14 h after stimulation. Results are the mean ± SEM (n = 3). n.i.= no inhibition. * $p < 0.001$, † $p > 0.05$. L-NAME and Dexamethasone was used as positive controls.

Discussion

The anticancer and immunomodulatory effect of natural and synthetic xanthones have been extensible reported in literature (Pinto *et al.* 2005; Teixeira *et al.* 2005; Castanheiro *et al.* 2007; Obolskiy *et al.* 2009; Gutierrez-Orozco and Failla 2013). In particular, 1,2-DHX inhibited the gowth of several cancer cell lines and the proliferation of stimulated T lymphocytes (Pedro *et al.* 2002; Sousa *et al.* 2002). In spite of these insights, and as far as we know, no studies were performed considering the immune microenvironment of 1,2-DHX-conditionated macrophages mediated effect on melanoma cell.

In the present study, the effect of 1,2-DHX on A375-C5 melanoma cell line was evaluated. However, a significant difference of potency was observed when comparing the GI_{50} fot A375-C5 (GI_{50} = 55.0 ± 2.3 µM) and UACC-62 melanoma cell line (GI_{50} = 14.0 ± 0.3 µM), this may be explained by the difference morphology of the cell, i.e. epithelial (A375-C5) and non-epethelial (UACC-62).

Melanoma is a highly immunogenic tumor (Hussein 2004), therefore it was hyphotesised the possible improvement of anticancer capacity of 1,2-DHX by immune system involvement. It was showed that 1,2-DHX strongly interfere with macrophages immune microenvironment, resulting in a two-fold increase of the cytotoxic effect of macrophages on A375-C5 melanoma cells. This finds indicates a possible induction of an antitumor macrophages phenotype by 1,2-DHX since supernatants of non-treated macrophages by it self showed much lower inhibition comparatively to treated macrophages.

In order to certify the underlying mechanism mediated by 1,2-DHX in macrophages, the expression of cytokines, namely interleukin - 1β (IL-1β) and tumor necrosis factor - α (TNF- α) (characteristic of a M1 phenotype) and tumor grow factor - β1 (TGF-β1) and IL-10 (characteristic of a M2-like phenotype) was evaluated.

Macrophages are dynamic and heterogeneous cells mainly due to their capacity to respond to stimulus. According to the microenvironment they may be polarized into a spectrum of phenotypes ranging from the pro-inflammatory M1 (classic) to the immunossupressive M2 (alternative) (Allavena *et al.* 2008; Biswas *et al.* 2013). Several evidences indicate that macrophages phenotypes can change during tumor progression (Zaynagetdinov *et al.* 2011). M1 activation may induce chronic inflammation, a factor that could predispose to tumor initiation (Greten *et al.* 2004; Pikarsky *et al.* 2004). However, in early stages of tumor progression, TAMs adopt a M1-like phenotype that contributes to tumor immunity. M2 phenotype is mainly expressed in established tumors and induce immunosuppressive, angiogenic and metastatic effects (Mantovani *et al.* 2004; Sica *et al.* 2006; Gordon and Mantovani 2011; Zaynagetdinov *et al.* 2011; Liao *et al.* 2014).

1,2-DHX suppress the expression of IL-1β to concentrations similar to basal and stimulated TNF-α expression. It also inhibited IL-10 production and stimulated TGF-β1 expression but only at concentration above GI_{50} (100 μM), while any alteration was detected at lower concentration.

IL-1β, a pro-inflammatory cytokine mainly produced by monocytes and macrophages is an example of the pleiotropism of immune system. In melanoma its expression was associated to tumor progression and promotion of lung metastases from melanoma (Giavazzi *et al.* 1990; Meyer *et al.* 2011). Therefore, IL-1β has been associated with all steps of malignancy (carcinogenesis, progression, invasion and metastasis) and may even be expressed by the tumor cells (Apte and Voronov 2008). In contrast, it induces an immune response against malignant cells associated to the M1 macrophages phenotype (Fairweather and Cihakova 2009).

TNF-α stimulation predicts a favorable outcome in melanoma treatment since several reports associated the use of TNF inhibitors as a factor that appear to increase the risk of skin cancer, including melanoma (Mariette *et al.* 2011; Kouklakis *et al.* 2013). TNF-α expression by THP-1 cells at basal and LPS-stimulated level was significantly lower when compared to 1,2-DHX treated cells.

IL-10 and TGF-β1 are anti-inflammatory cytokine involved in carcinogenisis process. In melanoma, IL-10 was associated with metastatic formation (García-Hernández *et al.* 2002; Itakura *et al.* 2011) and TGF-β1 are highly expressed and increase in parallel with tumor progression (Krasagakis *et al.* 1998; Javelaud *et al.* 2008). In spite of the differences observed, the antitumor effect of 1,2-DHX conditioned macrophages medium was not due neither to IL-10 nor TGF-β1 expression, since for treatment with 50 μM of 1,2-DHX no significantly differences were observed for the expression of these cytokines by THP-1 macrophages.

Production of nitric oxide (NO) by macrophages in tumors has controversial funtions. It was reported that below a critical concentration of NO, it causes DNA mutations (Wink *et al.* 1998), inhibits apoptosis (Choi *et al.* 2002), promotes angiogenesis (Ziche and Morbidelli 2000), limits immune response against cancer (Wink *et al.* 1991) and promotes metastasis (Lala and Orucevic 1998). When it exceeds the critical concentration, NO induce apoptosis and suppress the growth of the tumor (Choudhari *et al.* 2013). NO has been reported as cytotoxic in melanoma and many other tumors, but simultaneously it modulates many pro-tumor pathways including resistance to apoptosis, cell cycle progression, angiogenesis invasion, and metastasis (Ying and Hofseth 2007; Choudhari *et al.* 2013). In order to clarify this relation and better understand the mechanism mediated by 1,2-DHX in macrophages, production of NO by macrophages was evaluated.

In NO production assay, RAW 264.7 was used instead of THP-1 human macrophages cell line since this was referred as an appropriet model to study iNOS system (Dirsch *et al.* 1998; Shih *et al.* 2010). Human macrophages *in vitro* produce low quantitie of NO in response to stimuli (Thomassen and Kavuru 2001). However either *in vitro* murine cells as *in vivo* human macrophages express great quantities of NO in inflammatory conditions (Ochoa *et al.* 1991; Dirsch *et al.* 1998)

1,2-DHX is a potent inhibitor of NO production by LPS-stimulated RAW 264.7 murine macrophages cell line (IC_{50} = 22.0 ± 0.9 µM). This effect was not due to cell death or to NO scavenging, thus it was proved that interference was at the level of NO production.

In stimulated macrophages, NO is produced by the inducible form of NO synthetase (iNOS or NOS2) from L-arginine and molecular oxygen (Gross and Wolin 1995; Aramaki 2000). *NOS2* gene regulation, as the majority of genes may occur at transcriptional and post-transcriptional level (Nathan and Xie 1994; Bogdan *et al.* 2000; Brunet 2001).

With the purpose of evaluate the time point when the compound interfere with the enzyme to inhibit NO production, xanthone was added at different hours: 0h (simultaneously), 6h and 14h after RAW 264.7 macrophages stimulation with LPS. Although, 1,2-DHX added simultaneously has potent inhibition; when it was added 6h after stimulation the effect decreased significantly ($p < 0.001$) and when added after 14h, the effect was nule. This finds leads us to conclude that the compound may exerts their action by interfering with iNOS expression, once this enzyme is transcripted within 2-4 hours and translated within six hours after LPS-stimulation in macrophages (Xie et al. 1994). L-NAME (inhibitor of iNOS activity) (McCall *et al.* 1991), and Dexamethasone, inhibitor of iNOS expression (Korhonen *et al.* 2002) was used as positive controls and the obtained results was in accordance with the mechanism proposed to these inhibitors by our technique.

Inhibition of iNOS indicated an anti-inflamatory potential of 1,2-DHX. However, inflammation and NO production has controversial effects in cancer. NO has been reported as cytotoxic in melanoma and many other tumors, but simultaneously it modulates many pro-tumor pathways including resistance to apoptosis, cell cycle progression, angiogenesis, invasion and metastasis (Ying and Hofseth 2007; Choudhari *et al.* 2013).

The underling mechanism of inhibition of iNOS expression by 1,2-DHX may be explained by the stimulation of TGF-β1. This cytokine has been reported as a destabilizer of iNOS mRNA avoiding gene translation and inhibiting NO production. Involvement of NF-κB pathway may also be involved since it contributes to iNOS synthesis and upregulate IL-1β level and 1,2-DHX treated THP-1 macrophages expressed either lower IL-1β and NO levels comparatively to control cells. This finds leads us to hypothesize the

interference of the xanthone with NF-κB pathway. More studies must be performed in order to corroborate this supposition.

The set of all data find in this study allowed to infer a potential of 1,2-DHX in melanoma treatment, due to its cytotoxic activity through direct inhibition of cell growth and by modulation of macrophages immune microenvironment. Characteristics of 1,2-DHX in melanoma are even more important taking in account the lack of appropriated therapeutic alternatives in this tumor. However, further studies are needed to attest this relation *in vivo*.

References

Allavena P, Sica A, Solinas G *et al* (2008) The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. Crit Rev Oncol Hematol 66: 1-9.

Apte RN & Voronov E (2008) Is interleukin-1 a good or bad 'guy' in tumor immunobiology and immunotherapy? Immunological Reviews 222: 222-241.

Aramaki Y (2000) Liposomes as immunomodulator--inhibitory effect of liposomes on NO production from macrophages. Biol Pharm Bull 23: 1267-74.

Bernengo MG, Quaglino P, Cappello N *et al* (2000) Macrophage-mediated immunostimulation modulates therapeutic efficacy of interleukin-2 based chemoimmunotherapy in advanced metastatic melanoma patients. Melanoma Res 10: 55-65.

Biswas SK, Allavena P & Mantovani A (2013) Tumor-associated macrophages: functional diversity, clinical significance, and open questions. Semin Immunopathol 35: 585-600.

Bogdan C, Rollinghoff M & Diefenbach A (2000) Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Curr Opin Immunol 12: 64-76.

Brocker EB, Zwadlo G, Holzmann B *et al* (1988) Inflammatory cell infiltrates in human melanoma at different stages of tumor progression. Int J Cancer 41: 562-7.

Brunet LR (2001) Nitric oxide in parasitic infections. Int Immunopharmacol 1: 1457-67.

Castanheiro RaP, Pinto MMM, Silva AMS *et al* (2007) Dihydroxyxanthones prenylated derivatives: synthesis, structure elucidation, and growth inhibitory activity on human tumor cell lines with improvement of selectivity for MCF-7. Bioorganic & medicinal chemistry 15: 6080-8.

Cerqueira F, Cidade H, Van Ufford L *et al* (2008) The natural prenylated flavone artelastin is an inhibitor of ROS and NO production. Int Immunopharmacol 8: 597-602.

Cerqueira F, Cordeiro-Da-Silva A, Araujo N *et al* (2003) Inhibition of lymphocyte proliferation by prenylated flavones: artelastin as a potent inhibitor. Life Sci 73: 2321-34.

Chanput W, Mes JJ & Wichers HJ (2014) THP-1 cell line: An in vitro cell model for immune modulation approach. Int Immunopharmacol.

Chanput W, Reitsma M, Kleinjans L *et al* (2012) beta-Glucans are involved in immune-modulation of THP-1 macrophages. Mol Nutr Food Res 56: 822-33.

Chen P, Huang Y, Bong R *et al* (2011) Tumor-associated macrophages promote angiogenesis and melanoma growth via adrenomedullin in a paracrine and autocrine manner. Clinical cancer research : an official journal of the American Association for Cancer Research 17: 7230-9.

Choi BM, Pae HO, Jang SI *et al* (2002) Nitric oxide as a pro-apoptotic as well as anti-apoptotic modulator. J Biochem Mol Biol 35: 116-26.

Choudhari SK, Chaudhary M, Bagde S *et al* (2013) Nitric oxide and cancer: a review. World J Surg Oncol 11: 118.

Dirsch VM, Stuppner H & Vollmar AM (1998) The Griess assay: suitable for a bio-guided fractionation of anti-inflammatory plant extracts? Planta Med 64: 423-6.

Dranoff G (2009) Targets of protective tumor immunity. Ann N Y Acad Sci 1174: 74-80.

Fairweather D & Cihakova D (2009) Alternatively activated macrophages in infection and autoimmunity. J Autoimmun 33: 222-30.

Gales L, Sousa MED, Pinto MMM *et al* (2001) Naturally occurring 1,2,8-trimethoxyxanthone and biphenyl ether intermediates leading to 1,2-dimethoxyxanthone. Acta Crystallographica Section C 57: 1319-1323.

García-Hernández ML, Hernández-Pando R, Gariglio P *et al* (2002) Interleukin-10 promotes B16-melanoma growth by inhibition of macrophage functions and induction of tumour and vascular cell proliferation. Immunology 105: 231-243.

Gast A, Bermejo JL, Claus R *et al* (2011) Association of inherited variation in Toll-like receptor genes with malignant melanoma susceptibility and survival. PLoS One 6: e24370.

Giavazzi R, Garofalo A, Bani MR *et al* (1990) Interleukin 1-induced augmentation of experimental metastases from a human melanoma in nude mice. Cancer Res 50: 4771-5.

Gordon S & Mantovani A (2011) Diversity and plasticity of mononuclear phagocytes. Eur J Immunol 41: 2470-2.

Gottlieb OR, Mesquita AaL, De Oliveira GG *et al* (1970) Xanthones from Kielmeyera speciosa. Phytochemistry 9: 2537-2544.

Graziani G, Tentori L & Navarra P (2012) Ipilimumab: A novel immunostimulatory monoclonal antibody for the treatment of cancer. Pharmacological Research 65: 9-22.

Greten FR, Eckmann L, Greten TF *et al* (2004) IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell 118: 285-96.

Gross SS & Wolin MS (1995) Nitric oxide: pathophysiological mechanisms. Annu Rev Physiol 57: 737-69.

Gupta S, Rodrigues LM, Esteves AP *et al* (2008) Synthesis of N-aryl-5-amino-4-cyanopyrazole derivatives as potent xanthine oxidase inhibitors. Eur J Med Chem 43: 771-80.

Gutierrez-Orozco F & Failla ML (2013) Biological activities and bioavailability of mangosteen xanthones: a critical review of the current evidence. Nutrients 5: 3163-83.

He X, Shu J, Xu L *et al* (2012) Inhibitory effect of Astragalus polysaccharides on lipopolysaccharide-induced TNF-a and IL-1beta production in THP-1 cells. Molecules 17: 3155-64.

Hodi FS, O'day SJ, Mcdermott DF *et al* (2010) Improved Survival with Ipilimumab in Patients with Metastatic Melanoma. New England Journal of Medicine 363: 711-723.

Hussein MR (2004) Genetic pathways to melanoma tumorigenesis. J Clin Pathol 57: 797-801.

Itakura E, Huang RR, Wen DR *et al* (2011) IL-10 expression by primary tumor cells correlates with melanoma progression from radial to vertical growth phase and development of metastatic competence. Mod Pathol 24: 801-9.

Javelaud D, Alexaki VI & Mauviel A (2008) Transforming growth factor-beta in cutaneous melanoma. Pigment Cell Melanoma Res 21: 123-32.

Korhonen R, Lahti A, Hamalainen M *et al* (2002) Dexamethasone inhibits inducible nitric-oxide synthase expression and nitric oxide production by destabilizing mRNA in lipopolysaccharide-treated macrophages. Mol Pharmacol 62: 698-704.

Kouklakis G, Efremidou EI, Pitiakoudis M *et al* (2013) Development of primary malignant melanoma during treatment with a TNF-alpha antagonist for severe Crohn's disease: a case report and review of the hypothetical association between TNF-alpha blockers and cancer. Drug Des Devel Ther 7: 195-9.

Krasagakis K, Tholke D, Farthmann B *et al* (1998) Elevated plasma levels of transforming growth factor (TGF)-beta1 and TGF-beta2 in patients with disseminated malignant melanoma. Br J Cancer 77: 1492-4.

Kuphal S & Bosserhoff A (2009) Recent progress in understanding the pathology of malignant melanoma. J Pathol 219: 400-9.

Lala PK & Orucevic A (1998) Role of nitric oxide in tumor progression: lessons from experimental tumors. Cancer Metastasis Rev 17: 91-106.

Liao Q, Zeng Z, Guo X *et al* (2014) LPLUNC1 suppresses IL-6-induced nasopharyngeal carcinoma cell proliferation via inhibiting the Stat3 activation. Oncogene 33: 2098-109.

Makitie T, Summanen P, Tarkkanen A *et al* (2001) Tumor-infiltrating macrophages (CD68(+) cells) and prognosis in malignant uveal melanoma. Invest Ophthalmol Vis Sci 42: 1414-21.

Mantovani A, Allavena P & Sica A (2004) Tumour-associated macrophages as a prototypic type II polarised phagocyte population: role in tumour progression. Eur J Cancer 40: 1660-7.

Mantovani A & Sica A (2010) Macrophages, innate immunity and cancer: balance, tolerance, and diversity. Curr Opin Immunol 22: 231-7.

Mariette X, Matucci-Cerinic M, Pavelka K *et al* (2011) Malignancies associated with tumour necrosis factor inhibitors in registries and prospective observational studies: a systematic review and meta-analysis. Ann Rheum Dis 70: 1895-904.

Mccall TB, Feelisch M, Palmer RMJ *et al* (1991) Identification of N-iminoethyl-l-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells. British Journal of Pharmacology 102: 234-238.

Meyer C, Sevko A, Ramacher M *et al* (2011) Chronic inflammation promotes myeloid-derived suppressor cell activation blocking antitumor immunity in transgenic mouse melanoma model. Proc Natl Acad Sci U S A 108: 17111-6.

Monks A, Scudiero D, Skehan P *et al* (1991) Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. J Natl Cancer Inst 83: 757-66.

Nathan C & Xie QW (1994) Regulation of biosynthesis of nitric oxide. J Biol Chem 269: 13725-8.

Obolskiy D, Pischel I, Siriwatanametanon N *et al* (2009) Garcinia mangostana L.: a phytochemical and pharmacological review. Phytother Res 23: 1047-65.

Ochoa JB, Udekwu AO, Billiar TR *et al* (1991) Nitrogen oxide levels in patients after trauma and during sepsis. Ann Surg 214: 621-6.

Pedro M, Cerqueira F, Sousa ME *et al* (2002) Xanthones as inhibitors of growth of human cancer cell lines and their effects on the proliferation of human lymphocytes in vitro. Bioorganic & medicinal chemistry 10: 3725-30.

Pikarsky E, Porat RM, Stein I *et al* (2004) NF-kappaB functions as a tumour promoter in inflammation-associated cancer. Nature 431: 461-6.

Pinto M & Castanheiro R (2009) Natural Prenylated Xanthones: Chemistry and Biological Activities. In*:* BRAHMACHARI, G. (ed.) Natural Products: Chemistry, Biochemistry and Pharmacology. Oxford, UK: Alpha science International.

Pinto MM, Sousa ME & Nascimento MS (2005) Xanthone derivatives: new insights in biological activities. Curr Med Chem 12: 2517-38.

Porta C, Subhra Kumar B, Larghi P *et al* (2007) Tumor promotion by tumor-associated macrophages. Adv Exp Med Biol 604: 67-86.

Qian BZ & Pollard JW (2010) Macrophage diversity enhances tumor progression and metastasis. Cell 141: 39-51.

Raaijmakers MI, Rozati S, Goldinger SM *et al* (2013) Melanoma immunotherapy: historical precedents, recent successes and future prospects. Immunotherapy 5: 169-82.

Shih MF, Cheng YD, Shen CR *et al* (2010) A molecular pharmacology study into the anti-inflammatory actions of Euphorbia hirta L. on the LPS-induced RAW 264.7 cells through selective iNOS protein inhibition. J Nat Med 64: 330-5.

Sica A, Schioppa T, Mantovani A *et al* (2006) Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. Eur J Cancer 42: 717-27.

Solinas G, Germano G, Mantovani A *et al* (2009) Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. J Leukoc Biol 86: 1065-73.

Solit DB & Rosen N (2011) Resistance to BRAF Inhibition in Melanomas. New England Journal of Medicine 364: 772-774.

Sousa EP, Silva AMS, Pinto MMM *et al* (2002) Isomeric Kielcorins and Dihydroxyxanthones: Synthesis, Structure Elucidation, and Inhibitory Activities of Growth of Human Cancer Cell Lines and on the Proliferation of Human Lymphocytes In Vitro. Helvetica Chimica Acta 85: 2862-2876.

Teixeira M, Cerqueira F, Barbosa CM *et al* (2005) Improvement of the inhibitory effect of xanthones on NO production by encapsulation in PLGA nanocapsules. J Drug Target 13: 129-35.

Thomassen MJ & Kavuru MS (2001) Human alveolar macrophages and monocytes as a source and target for nitric oxide. Int Immunopharmacol 1: 1479-90.

Varney ML, Johansson SL & Singh RK (2005) Tumour-associated macrophage infiltration, neovascularization and aggressiveness in malignant melanoma: role of monocyte chemotactic protein-1 and vascular endothelial growth factor-A. Melanoma Res 15: 417-25.

Vermes B, Seligmann O & Wagner H (1985) Synthesis of xanthone O-glycosides. Part III. Synthesis of 1- and 8-O-β-D-glycosides of 5-O-methyl- and de-O-methylbellidifolin. Helvetica Chimica Acta 68: 2359-2366.

Wink DA, Kasprzak KS, Maragos CM *et al* (1991) DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. Science 254: 1001-3.

Wink DA, Vodovotz Y, Laval J *et al* (1998) The multifaceted roles of nitric oxide in cancer. Carcinogenesis 19: 711-21.

Xie QW, Kashiwabara Y & Nathan C (1994) Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. J Biol Chem 269: 4705-8.

Ying L & Hofseth LJ (2007) An emerging role for endothelial nitric oxide synthase in chronic inflammation and cancer. Cancer Res 67: 1407-10.

Zaynagetdinov R, Sherrill TP, Polosukhin VV *et al* (2011) A critical role for macrophages in promotion of urethane-induced lung carcinogenesis. J Immunol 187: 5703-11.

Ziche M & Morbidelli L (2000) Nitric oxide and angiogenesis. J Neurooncol 50: 139-48.

VII. Conclusions

The present study comprised the evaluation of antitumor and effect of two different xanthones: alpha-mangostin obtained from mangosteen and 1,2-dihydroxyxanthone obtained from synthesis, and their involvement in several parameters of immune system, concerning their anticancer activity. Interference of α-MG was tested in cells of innate and adaptive immune system, while 1,2-DHX was only evaluated in macrophages due to recent studies in lymphocytes performed by our group.

The main conclusions from the results of this study are as followed:

Antitumor activity of compounds:

- α-MG and 1,2-DHX showed to be good inhibitors of A375-C5 melanoma cell line growth. However, the potency of the cytotoxic effect of α-MG was significantly higher than that of 1,2DHX.

Antitumor activity of macrophages conditioned culture medium:

- 1,2-DHX interfered with the immune macrophages-dependent microenvironment of A375-C5 melanoma cells;
- For α-MG this effect was not clearly observed, probably due to the doses tested;

Inhibition of NO production:

- Expression of NO by RAW 26.7 macrophages was suppressed after treatment with α-MG and 1,2-DHX and, once again, a more significant inhibition was obtained for α-MG.
- Inhibition of NO production by these compounds is probably a consequence of inhibition of *NOS2* gene expression, occurring just after stimulation.

Interference of cytokine expressed by THP-1 macrophages:

- α-MG and 1,2-DHX decreased the expression of IL-1β, a pro-inflammatory cytokine associated to carcinogenesis, progression, invasion and metastases, and either as mediator of immunosurveillance.
- α-MG also inhibit the anti-inflammatory cytokine ,TGF-β1, which may compromise the tumor immune scape and it could allow an immune response against tumor.
- At concentration above GI_{50}, 1,2-DHX suppressed IL-10 and stimulated TGF-β1, however it cannot be associated to antitumor effect of supernatants of treated macrophages since it was not verified at the concentration must closer GI_{50}.
- Stimulation of TGF-β1 by 1,2-DHX could explain the inhibitory effect against iNOS by this compound. This cytokine has been reported as a destabilizer of iNOS mRNA avoiding gene translation and inhibiting NO production.

- The inhibition of IL-1β mediated by α-MG may ilucidate the underlaing mechanism of inhibition of NO production since IL-1β stimulate NF-κB pathway that, in turn, activate the expression of iNOS enzyme.
- IL-10 and TNF-α concentrations did not alter in macrophages treated with α-MG comparatively to non-treated macrophages; while only TNF-α concentration remains stable in 1,2-DHX treated macrophages.

Antiproliferative activity against human lymphocytes:

- α-MG strongly inhibit prolipheration of human T lymphocytes from peripheral blood.
- α-MG also reduced the expression of IL-10 and TNF-α to basal levels in these cells, but only at concentration much higher than IC_{50} which indicates a interference in cytokines proliferation without affect cytokines expression.

In summary, this dissertation allows to infer the potential of alpha-mangostin and 1,2-dihydroxyxanthone xanthones in melanoma treatment due to their cytotoxic activity and suggestive effect in cancer immunotherapy.

Although, further studies are needed to attest and explain the mechanism of action of xanthones against melanoma.